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(54) Title: CALCIUM RECEPTOR-ACTIVE MOLECULES

(57) Abstract

Method and composition useful for treating a patient having a disease characterized by an abnormal level of one or more components, the activity of which is regulated or affected by activity of one or more inorganic-ion receptor. Novel compounds useful in these methods and compositions are also provided. The method includes administering to the patient a therapeutically effective amount of a molecule active at one or more inorganic-ion receptors as an agent or antagonist. Preferably, the molecule is able to act as either a selective agonist or antagonist at a Ca^{2+} receptor of one or more but not all cells chosen from the group consisting of parathyroid cells, bone osteoclasts, juxtaglomerular kidney cells, proximal tubule kidney cells, distal tubule kidney cell, cell of the thick ascending limb of Henle's loop and/or collecting duct, keratinocyte in the epidermis, parafollicular cell in the thyroid (C-cells), intestinal cell, trophoblast in the placenta, platelet, vascular smooth muscle cell, cardiac atrial cell, gastrin and glucagon secreting cells, kidney mesangial cell and mammary cell.

- 1 -

CALCIUM RECEPTOR-ACTIVE MOLECULES

Field of the Invention

This invention relates to the design, development, composition and use of novel calcimimetic molecules able to act in a manner analogous to extracellular calcium ions on cells, to calcilytic molecules which block the activity of extracellular calcium ions on cells, and to methods for their use and identification.

It also relates to a novel superfamily of inorganic-ion receptors which includes, among others, calcium receptors, nucleic acids encoding such receptors, cells, tissues and animals containing such nucleic acids, antibodies to receptors, assays utilizing receptors, and methods relating to all of the foregoing.

Background of the Invention

The following description provides a summary of information relevant to the present invention. It is not an admission that any of the information provided herein is prior art to the presently claimed invention, nor that any of the publications specifically or implicitly referenced

- 2 -

are prior art to that invention.

Certain cells in the body respond not only to chemical signals, but also to ions such as extracellular calcium ions (Ca^{2+}). Changes in the concentration of extracellular Ca^{2+} (referred to herein as "$[Ca^{2+}]$") alter the functional responses of these cells. One such specialized cell is the parathyroid cell which secretes parathyroid hormone (PTH). PTH is the principal endocrine factor regulating Ca^{2+} homeostasis in the blood and extracellular fluids.

PTH, by acting on bone and kidney cells, increases the level of Ca^{2+} in the blood. This increase in $[Ca^{2+}]$ then acts as a negative feedback signal,depressing PTH secretion. The reciprocal relationship between $[Ca^{2+}]$ and PTH secretion forms the essential mechanism maintaining bodily Ca^{2+} homeostasis.

Extracellular Ca^{2+} acts directly on the parathy roid cell to regulate PTH secretion. The existence of a parathyroid cell surface protein which detects changes in $[Ca^{2+}]$ has been suggested. This protein acts as a receptor for extracellular Ca^{2+} ("the Ca^{2+} receptor"), and is suggested to detect changes in $[Ca^{2+}]$ and to initiate a functional cellular response, PTH secretion. For example, the role of Ca^{2+} receptors and extracellular Ca^{2+} in the regulation of intracellular Ca^{2+} and cell function is reviewed in Nemeth et al., 11 <u>Cell Calcium</u> 319, 1990; the role of Ca^{2+} receptors in parafollicular and parathyroid cells is discussed in Nemeth, 11 <u>Cell Calcium</u> 323, 1990; and the role of Ca^{2+} receptors on bone osteoclasts is discussed by Zaidi, 10 <u>Bioscience Reports</u> 493, 1990.

- 3 -

Other cells in the body, specifically the osteoclast in bone, the juxtaglomerular, proximal tubule cells in the kidney, the keratinocyte in the epidermis, the parafollicular cell in the thyroid, intestinal cells the trophoblast in the placenta, have the capacity to sense changes in $[Ca^{2+}]$. It has been suggested that cell surface Ca^{2+} receptors may also be present on these cells, imparting to them the ability to detect and to initiate or enable a response to changes in $[Ca^{2+}]$.

In parathyroid cells, osteoclasts, parafollicular cells (C-cells), keratinocytes, juxtaglomerular cells and trophoblasts, an increase in $[Ca^{2+}]$ evokes an increase in intracellular free Ca^{2+} concentration ("$[Ca^{2+}]_i$"). Such an increase may be caused by influx of extracellular Ca^{2+} or by mobilization of Ca^{2+} from intracellular organelles. Changes in $[Ca^{2+}]_i$ are readily monitored and quantitated using fluorimetric indicators such as fura-2 or indo-1 (Molecular Probes, Eugene, OR). Measurement of $[Ca^{2+}]_i$ provides an assay to assess the ability of molecules to act as agonists or antagonists at the Ca^{2+} receptor.

In parathyroid cells, increases in the concentration of extracellular Ca^{2+} evoke rapid and transient increases in $[Ca^{2+}]_i$ which are followed by lower yet sustained increases in $[Ca^{2+}]_i$. The transient increases in $[Ca^{2+}]_i$ arise from the mobilization of intracellular Ca^{2+}, whereas the lower, sustained increases result from the influx of extracellular Ca^{2+}. The mobilization of intracellular Ca^{2+} is accompanied by increased formation of inositol-1,4,5-trisphosphate (IP_3)

- 4 -

and diacylglycerol, two biochemical indicators which are
associated with receptor-dependent mobilization of
intracellular Ca^{2+} in various other cells.

In addition to Ca^{2+}, various other di- and
trivalent cations, such as Mg^{2+}, Sr^{2+}, Ba^{2+}, La^{3+},
and Gd^{3+} also cause the mobilization of intracellular
Ca^{2+} in parathyroid cells. Mg^{2+} and La^{3+} also
increase the forma tion of IP_3; all these inorganic
cations depress the secretion of PTH. The postulated Ca^{2+}
receptor on the parathyroid cell is therefore promiscuous
because it detects a variety of extracellular di- and
trivalent cations.

The ability of various compounds to mimic
extracellular Ca^{2+} in vitro is discussed by Nemeth et al.,
(spermine and spermidine) in "Calcium-Binding Proteins in
Health and Disease", 1987, Academic Press, Inc., pp. 33-35;
Brown et al., (e.g., neomycin) 128 Endocrinology 3047, 1991;
Chen et al., (diltiazem and its analog, TA-3090) 5 J. Bone
and Mineral Res. 581, 1990; and Zaidi et al., (verapamil)
167 Biochem Biophys Res Comm 807, 1990.

Brown et al., 6 J. Bone and Mineral Res. 11, 1991
discuss the existing theories regarding the effects of
Ca^{2+} ions on parathyroid cells, and propose that the
results may be explained by both a receptor-like mechanism
and a receptor-independent mechanism as follows:

> Polyvalent cations [e.g., divalent and
> trivalent cations] exert a variety of
> effects on parathyroid function, such as
> inhibition of parathyroid hormone (PTH)
> secretion and cAMP accumulation, stimulation
> of the accumulation of inositol phosphates,
> and elevation of the cytosolic calcium

- 5 -

concentration. These actions are thought to
be mediated through a "receptor-like"
mechanism. The inhibition of
agonist-stimulated cAMP accumulation by
divalent and trivalent cations, for example,
is blocked following preincubation with
pertussis toxin. Thus, the putative
polyvalent cation receptor may be coupled to
inhibition of adenylate cyclase by the
inhibitory guanine nucleotide regulatory (G)
protein, G_i.

We recently showed that the
polycationic antibiotic, neomycin, mimics
the actions of di- and trivalent cations in
several aspects of parathyroid function.
To determine whether these actions were
specific to this agent or represented a more
generalized action of polycations, we tested
the effects of the highly basic peptides,
polyarginine and polylysine, as well as
protamine on the same parameters in
dispersed bovine parathyroid cells. The
results demonstrate that the parathyroid
cell responds to a variety of polycations as
well as to polyvalent cations, potentially
via similar biochemical pathways. These
results are discussed in terms of the
recently postulated, "receptor-independent"
modulation of G proteins by polycations in
other systems.

*   *   *

The Ca^{2+} receptor has been presumed
to be analogous to other G protein-coupled
receptors [e.g., a glycoprotein], but recent
studies with other cell types have raised
the possibility that polycations can
modulate cell function by alternative or
additional mechanisms. In mast cells, for
example, a variety of amphipathic cations,
including mastoparan, a peptide from wasp
venom, 48/80, a synthetic polycation, and
polylysine, enhance secretion by a pertussis

- 6 -

toxin-sensitive mechanism, suggesting the
involvement of a G protein. No classic cell
surface receptor has been identified that
could mediate the actions of these diverse
agents. Furthermore, these same compounds
have been shown to activate directly
purified G proteins in solution or in
artificial phospholipid vesicles. On the
basis of these observations, it has been
proposed that amphipathic cations activate G
proteins and, in turn, mast cell secretion
by a "receptor-independent" mechanism.

    Polycations have also been shown to
interact strongly with acidic
phospholipids. Polylysines of varying chain
lengths (20-1000 amino acids) bind to
artificial phospholipid vesi cles with
dissociation constants in the range of
0.5 nM to 1.5 μM. The binding affinity is
directly related to the length of the
polylysine chain, with polymers of 1000
amino acids having a K_d of 0.5 nM, shorter
polymers having higher Kd values, and lysine
not interacting to a significant extent.
This relationship between potency and chain
length is similar to that observed for the
effects of polylysine $_{10,200}$, polylysine
$_{3800}$, and lysine on parathyroid function.

    It is possible that the binding of
polycations to biomembranes produces some of
their biologic actions. The
permeabilization of the plasma membrane
induced in some cell types by a variety of
pore-forming agents, including polycations,
has been postulated to be mediated by their
interaction with a phosph atidylserine-like
structure. In addition, the
"receptor-independent" activation of
purified G proteins by amphipathic cations
is potentiated when these proteins are
incorporated into phospholipid vesicles.

    Calcium ions, in the millimolar
concentration range, also produce marked
changes in membrane structure. In some
cases, calcium can either antagonize or

- 7 -

potentiate the interaction of polycations
with membrane lipids. These considerations
raise the possibility that the actions of
both polyvalent cations and polycations on
parathyroid cells could involve a
receptor-independent mechanism not requiring
the presence of a classic, cell surface, G
protein-coupled receptor. Further studies,
however, are required to elucidate the
molecular basis for Ca^{2+} sensing by this
and other cell types. [Citations omitted.]

Shoback and Chen (6 (Supplement 1), <u>J. Bone and
Mineral Res.</u> 1991, S135) and Racke et al. (6 (Supplement 1),
<u>J. Bone and Mineral Res.</u> 1991, S118) describe experiments
which are said to indicate that a Ca^{2+} receptor or Ca^{2+}
sensor is present in parathyroid cells. Messenger RNA
isolated from such cells can be expressed in oocytes and
caused to provide those oocytes with a phenotype which might
be explained by the presence of a Ca^{2+} receptor protein.

<u>Summary of the Invention</u>

Applicant has demonstrated that Ca^{2+} receptor
proteins enable certain specialized cells involved in bodily
Ca^{2+} metabolism to detect and respond to changes in the
concentration of extracellular Ca^{2+}. Although these
receptors share certain general characteristics, they can be
selectively affected by different pharmacological agents.
As detailed below, certain molecules are identified with
selective activity on Ca^{2+} receptors at parathyroid cells,
osteoclasts, and C-cells.

Ca^{2+} receptors constitute discrete molecular
targets for a new class of molecules that mimic
("calcimimetics") or antagonize ("calcilytics") the actions
of extracellular Ca^{2+}. Such receptors are present on cell

- 8 -

surfaces and have a low affinity for extracellular Ca^{2+} (apparent K_d generally greater than about 0.5 mM). Such receptors may include a free or bound effector mechanism, as defined by Cooper, Bloom and Roth, "The Biochemical Basis of Neuropharmacology", Ch. 4. Such receptors are thus distinct from intracellular Ca^{2+} receptors, e.g., calmodulin and the troponins. Calcimimetics, for example, act on Ca^{2+} receptors selectively to directly or indirectly depress the function of parathyroid cells or osteoclasts or to stimulate the function of C-cells. Calcimimetics and calcilytics of this invention allow novel therapies for hyperparathyroidism, osteoporosis and other Ca^{2+}-related diseases. This application concerns in one aspect targeting Ca^{2+} receptors on each of these three cell types and other cell types that detect and respond to changes in $[Ca^{2+}]$.

Applicant is the first to demonstrate a Ca^{2+} receptor protein in parathyroid cells, and to pharmaco-logically differentiate such Ca^{2+} receptors in other cells, such as C-cells and osteoclasts. Applicant is also the first to describe methods by which molecules active at these Ca^{2+} receptors can be identified and used as lead molecules in the discovery, development, design, modification and/or construction of useful calcimimetics or calcilytics which are active at Ca^{2+} receptors. Such calcimimetics or calcilytics are useful in the treatment of various disease states characterized by abnormal levels of one or more components, e.g., polypeptides such as hormones, enzymes or growth factors, the expression and/or secretion of which is regulated or affected by activity at one or more Ca^{2+} receptors. Further, the identification of different

- 9 -

Ca^{2+} receptors in different cell types, and the specific
response of such receptors to different lead molecules
allows design and construction of specific molecules active
in treatment of specific diseases which can be affected by
action at such specific Ca^{2+} receptors. For example,
abnormal levels of parathyroid hormone secretion can be
affected by such specific molecules without affecting the
level of secretion of other Ca^{2+} regulated hormones and
the like.

Identification of such lead molecules was impeded
by the prior lack of a high-throughput screening system to
discover active molecules, and the absence of a structural
data base upon which to design effective drug candidates.
These barriers are now removed by cloning the parathyroid
cell Ca^{2+} receptor and functionally related receptors, and
systematically examining the structural features of certain
lead molecules that activate such cloned Ca^{2+} receptors
and functionally related receptors. Cloning of the Ca^{2+}
receptor also enables development of transfected cell lines
suitable for high-throughput screening of natural product or
molecule libraries and synthetic molecules. This, together
with structure-activity studies discussed below, provides
the technology necessary to develop novel calcimimetics and
calcilytics.

Applicant enables such procedures in this
application. A bovine parathyroid cell calcium receptor
cDNA has been cloned and is deposited in the ATCC under
accession number ATCC 75416. Using this clone,
inorganic-ion receptors in other tissues and species
homologs are easily obtained. For example, the human
parathyroid cell Ca^{2+}

- 10 -

receptor cDNA can be cloned by screening nucleic acid
libraries or by screening for functional expression in
<u>Xenopus</u> oocytes, and the structural features of organic
molecules necessary for activity on the Ca^{2+} receptor can
be determined through the testing of selected natural
products or other molecule libraries and subsequent
structure-activity studies.

Thus, in a first aspect, the invention features a
pharmaceutical composition including a molecule which either
mimics the activity of extracellular Ca^{2+} by evoking an
increase in $[Ca^{2+}]_i$ in a cell, or blocks an increase in
$[Ca^{2+}]_i$ elicited by extracellular Ca^{2+}. The molecule
has an EC_{50} of less than or equal to 5 µM, and is not
protamine.

By "mimic" is meant that the molecule has one or
more of the specific actions of extracellular Ca^{2+} on an
extracellular Ca^{2+} responsive cell. The term does not
require that all of the biological functions of
extracellular Ca^{2+} are mimicked, but rather that at least
one such function is mimicked. In addition it does not
require that the molecule bind to the same site on the
Ca^{2+} receptor as does extracellular Ca^{2+} (see for
example, the novel compound NPS 467 and its action in
Example 20 below). By "block" is meant that one such action
of Ca^{2+} is reduced or prevented by the molecule. The
EC_{50} can be determined in assays as described below, where
the activity mimicked is measured and the concentration of
molecule which mimics at half the maximum mimicking effect
is the EC_{50}. Conversely, the IC_{50} of a calcilytic is
that amount which blocks half maximal activity. Preferably,

- 11 -

such assays measure $[Ca^{2+}]_i$ increases and are confirmed
to be specific to a Ca^{2+} receptor by methods described
below, or their equivalent.

In preferred embodiments, bioassays described
herein demonstrate that the increase in $[Ca^{2+}]_i$ in a
cell is transient, having a duration of less than one
minute, and the increase in $[Ca^{2+}]_i$ is rapid, occurring
within thirty seconds; and the molecule also (a) evokes a
sus tained increase (greater than thirty seconds) in
$[Ca^{2+}]_i$, (b) evokes an increase in
inositol-1,4,5-trisphosphate and/or diacylglycerol levels,
e.g., within less than 60 seconds, and (c) inhibits
dopamine- or isoproterenol- stimulated cyclic AMP
formation. In addition, the tran sient increase in
$[Ca^{2+}]_i$ is abolished by pretreatment of the cell for ten
minutes with 10 mM sodium fluoride, or the transient
increase is diminished by brief pretreatment (not more than
ten minutes) of the cell with an activator of protein kinase
C, e.g., phorbol myristate acetate (PMA), mezerein or
(-)indolactam V.

In a parathyroid cell, those molecules which are
active in all of the assays described above are particularly
useful in this invention since they are specific in their
actions to a Ca^{2+} receptor of such a cell. This is
particularly true for the PMA pretreatment effect described
above.

In a more preferred embodiment, the cell is a
parathyroid cell, and the molecule inhibits parathyroid
hormone secretion from the cell. Other preferred
embodiments include molecules that elicit an increase in

$[Ca^{2+}]_i$ as detected, for example, as an increase in
Cl^- current in a Xenopus oocyte injected with mRNA from a
parathyroid cell, bone osteoclast, juxtaglomerular kidney
cell, proximal tubule kidney cell, distal tubule kidney
cell, cell of the thick ascending limb of Henle's loop
and/or collecting duct, keratinocyte in the epidermis,
parafollicular cell in the thyroid (C-cells), intestinal
cell, trophoblast in the placenta, platelet, vascular smooth
muscle cell, cardiac atrial cell, gastrin and glucagon
secreting cells, kidney mesangial cell and mammary cell.

In other preferred embodiments, the molecule
evokes the mobilization of intracellular Ca^{2+} to cause the
increase in $[Ca^{2+}]_i$; the cell is a C-cell or an
osteoclast and the molecule inhibits bone resorption in
vivo; the cell is an osteoclast and the molecule inhibits
bone resorption in vitro; or the cell is a C- cell and the
molecule stimulates calcitonin secretion in vitro or in
vivo; and most preferably the molecule is either a
calcimimetic or calcilytic having an EC_{50} or IC_{50} at a
Ca^{2+} receptor of less than or equal to 5 μM, and even
more preferably less than or equal to 1 μM, 100 nmolar,
10 nmolar, or 1 nmolar. Such lower EC_{50}'s or IC_{50}'s are
advantageous since they allow lower concentration of
molecules to be used in vivo or in vitro for therapy or
diagnosis. The discovery of molecules with such low
EC_{50}'s and IC_{50}'s enables the design and synthesis of
similarly potent and efficacious molecules.

By "calcimimetic" molecule is meant any molecule
which has one or more activities of extracellular Ca^{2+},
and preferably mimics the activity of Ca^{2+} at a Ca^{2+}

- 13 -

receptor. For example, when used in reference to a
parathyroid cell it is a molecule which, when tested on
parathyroid cells <u>in vitro</u>, possesses one or more, and
preferably all of the following characteristics as measured
by techniques well known to those in the art:

      1.   The molecule causes a rapid (time to peak < 5
sec) and transient increase in $[Ca^{2+}]_i$ that is
refractory to inhibition by 1 μM La^{3+} or Gd^{3+}. The
increase in $[Ca^{2+}]_i$ persists in the absence of extra
cellular Ca^{2+} but is abolished by pretreatment with
ionomycin (in the absence of extracellular Ca^{2+});

      2.   The molecule potentiates increases in
$[Ca^{2+}]_i$ elicited by submaximal concentrations of
extracellular Ca^{2+};

      3.   The increase in $[Ca^{2+}]_i$ elicited by
extracellular Ca^{2+} is not inhibited by dihydropyridines;

      4.   The transient increase in $[Ca^{2+}]_i$ caused
by the molecule is abolished by pretreatment for 10 min.
with 10 mM sodium fluoride;

      5.   The transient increase in $[Ca^{2+}]_i$ caused
by the molecule is diminished by pretreatment with an
activator of protein kinase C (PKC), such as phorbol
myristate acetate (PMA), mezerein or (-)-indolactam V. The
overall effect of the protein kinase C activator is to shift
the concentration-response curve of the molecule to the
right without affecting the maximal response;

      6.   The molecule causes a rapid (< 30 sec.)
increase in the formation of inositol-1,4,5- trisphosphate
and or diacylglycerol;

      7.   The molecule inhibits dopamine- or isopro

- 14 -

terenol-stimulated cyclic AMP formation;

     8. The molecule inhibits PTH secretion;

     9. Pretreatment with pertussis toxin (100 ng/ml for > 4 hrs.) blocks the inhibitory effect of the molecule on cyclic AMP formation but does not effect increases in $[Ca^{2+}]_i$, inositol-1,4,5-trisphosphate, or diacylglycerol, nor decreases in PTH secretion;

     10. The molecule elicits increases in $[Ca^{2+}]_i$ as detected, for example, as an increase in Cl^- current in <u>Xenopus</u> oocytes injected with poly(A)$^+$- enriched mRNA from bovine or human parathyroid cells but is without effect in <u>Xenopus</u> oocytes injected with water or rat brain or liver mRNA; and

     11. Similarly, using a cloned receptor from parathyroid cells, the molecule will elicit a response in <u>Xenopus</u> oocytes injected with the specific cDNA, mRNA or synthetic sense RNA (cRNA) encoding the receptor.

     By "calcilytic" molecule is meant any molecule which blocks one or more of the activities of extracellular Ca^{2+} on an extracellular Ca^{2+}-sensing cell, preferably by acting as an antagonist at the Ca^{2+} receptor. For example, when used in reference to a parathyroid cell, it is a molecule which, when tested on parathyroid cells <u>in vitro</u>, possesses one or more, and preferably all of the following characteristics as measured by techniques well known to those in the art:

     1. The molecule blocks, either partially or completely, the ability of increased concentrations of extracellular Ca^{2+} to:

     a) increase $[Ca^{2+}]_i$,

- 15 -

        b)   mobilize intracellular Ca^{2+},

        c)   increase the formation of inositol-
1,4,5-trisphosphate,

        d)   decrease dopamine- or isoproterenol-
stimulated cyclic AMP formation, and

        e)   inhibit PTH secretion;

    2.   At low $[Ca^{2+}]$, i.e., 0.5 mM, the molecule
by itself does not change $[Ca^{2+}]_i$;

    3.   The molecule blocks increases in Cl^-
current in <u>Xenopus</u> oocytes injected with poly(A)$^+$- mRNA
from bovine or human parathyroid cells elicited by
extracellular Ca^{2+} or calcimimetic compounds but not in
<u>Xenopus</u> oocytes injected with water or rat brain or liver
mRNA;

    4.   Similarly, using a cloned receptor from
parathyroid cells, the molecule will block a response in
<u>Xenopus</u> oocytes injected with the specific cDNA, mRNA or
cRNA encoding the Ca^{2+} receptor, elicited by extracellular
Ca^{2+} or a calcimimetic compound.

    Parallel definitions of useful calcimimetics and
calcilytics at Ca^{2+} receptors on other cell types are
evident from the examples provided below.

    The Ca^{2+} receptor is able to detect and respond to
certain inorganic polycations and polycationic organic
molecules. For example, the parathyroid cell is unable to
distinguish increases in extracellular Ca^{2+} concentration
from the addition of these organic polycations, presumably
because these organic molecules act just like extracellular
Ca^{2+} at the Ca^{2+} receptor. The calcimimetic molecules
of this invention are particularly good agonists of the

- 17 -

cell, cell of the thick ascending limb of Henle's loop
and/or collecting duct, keratinocyte in the epidermis,
parafollicular cell in the thyroid (C-cells), intestinal
cell, trophoblast in the placenta, platelet, vascular smooth
muscle cell, cardiac atrial cell, gastrin and glucagon
secreting cells, kidney mesangial cell and mammary cell.

It is the specificity of action of such molecules
that is particularly advantageous in this invention since it
allows specific _in vivo_ and _in vitro_ therapy and diagnosis
and discovery of additional calcimimetic or calcilytic
molecules.

In specific preferred embodiments, the molecule is
positively charged at physiological pH, and is selected from
the group consisting of branched or cyclic polyamines,
positively charged polyamino acids, and arylalkylamines,
_e.g._, the branched polyamine has the formula
$H_2N-(CH_2)_j-(NR_i-(CH_2)_j)_k-NH_2$ where k is an
integer from 1 to 10, each j is the same or different and is
an integer from 2 to 20, and each R_i is the same or
different and is selected from the group consisting of
hydrogen and $-(CH_2)_j-NH_2$, where j is as defined above,
and at least one R_i is not hydrogen.

In an alternative embodiment, the molecule has the
formula

$$X_m\text{—(Ar or R)}\diagdown \qquad\quad \overset{R}{\underset{|}{}}\quad \text{(Ar or R)-}X_m$$
$$\phantom{X_m\text{—(Ar or R)}}Y\text{—Z—N}{\Large\{}\begin{matrix}\text{—(R or X)}\\ \text{(R or X)}\end{matrix}$$
$$X_m\text{—(Ar or R)}\diagup$$
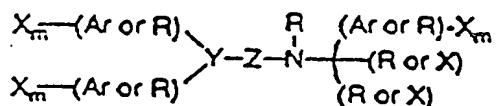
where each X independently is selected from the group

consisting of H, CH_3, CH_3O, CH_3CH_2O, Br, Cl, F,
CF_3, CHF_2, CH_2F, CF_3O, CH_3S, OH, CH_2OH, $CONH_2$,
CN, NO_2, and CH_3CH_2; Ar is a hydrophobic entity; each
R independently is selected from the group consisting of
hydrogen, methyl, ethyl, propyl, isopropyl, butyl, isobutyl,
cyclopentyl, cyclohexyl, cycloheptyl, cyclooctyl, indenyl,
indanyl, dihydroindolyl, thiodihydroindolyl, 2-, 3-, or 4-
piperid(in)yl; Y is selected from the group consisting of
CH, nitrogen and an unsaturated carbon; Z is selected from
the group consisting of oxygen, nitrogen, sulfur,

$$
\begin{array}{ccc}
\overset{\text{X or R}}{\underset{\text{X or R}}{\overset{|}{\underset{|}{C}}}} - \overset{\text{X or R}}{\underset{\text{X or R}}{\overset{|}{\underset{|}{C}}}} , &
\overset{\text{X or R}}{\underset{\text{X or R}}{\overset{|}{\underset{|}{C}}}} - \left(\overset{\text{X or R}}{\underset{\text{X or R}}{\overset{|}{\underset{|}{C}}}} - \right)_n \overset{\text{X or R}}{\underset{\text{X or R}}{\overset{|}{\underset{|}{C}}}} , & \overset{\text{X or R}}{\underset{\text{X or R}}{\overset{|}{\underset{|}{C}}}} \text{ and } \overset{\text{X or R}}{\underset{}{\text{CH}}} - (CH_2)_n -
\end{array}
$$
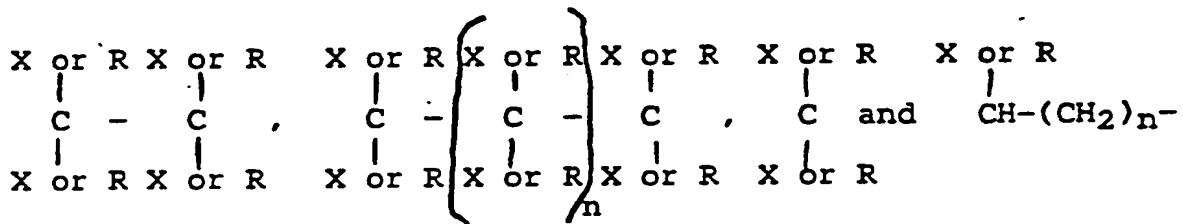
where each n is independently between 1 and 4 inclusive, and
each m is independently between 0 and 5 inclusive. Most
preferably the molecule is either a calcimimetic or calcilytic.
    In preferred embodiments, the hydrophobic entity is
selected from the group consisting of phenyl, 2-, 3-, or
4-pyridyl, 1- or 2-naphthyl, 1- or 2- quinolinyl, 2- or
3-indolyl, benzyl, and phenoxy; the molecule is an
R-phenylpropyl-α-phenethylamine derivative, and the molecule
has the formula:
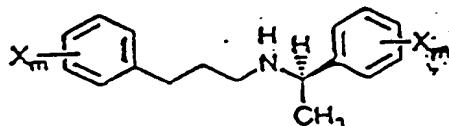
with each X preferably being independently selected from the
group consisting of Cl, F, CF_3, CH_3, and CH_3O.
    According to a preferred aspect of the present
invention, novel phenylpropyl-α-phenethylamine analogs and
derivatives are provided having the formula:

- 19 -
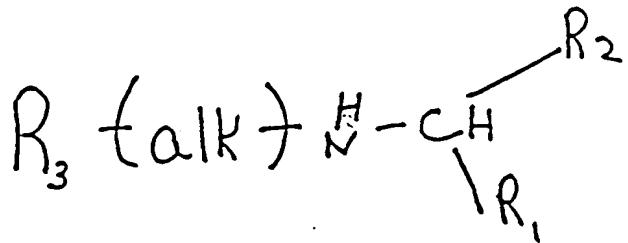
$$R_3 \left(alk\right) \overset{H}{N} - C\overset{R_2}{\underset{R_1}{H}}$$

wherein alk is straight or branched chain alkylene of from 1
to 6 carbon atoms;
R1 is lower alkyl of from 1 to 3 carbon atoms or lower haloalky
l of from 1 to 3 carbon atoms substituted with from 1 to 7 halog
en atoms; R and R are independently selected carbocyclic a
ryl or cycloalkyl groups, either monocyclic or bicyclic, having
5- or 6-membered rings
optionally substituted with 1 to 5 substituents independently
selected from lower alkyl of 1 to 3 carbon atoms, lower
haloalkyl of 1 to 3 carbon atoms substituted with 1 to 7
halogen atoms, lower alkoxy of 1 to 3 carbon atoms, halogen,
nitro, amino, alkylamino, amido, lower alkylamido of 1 to 3
carbon atoms, cyano, hydroxy, acyl of 2 to 4 carbon atoms
lower hydroxyalkyl of 1 to 3 carbon atoms or lower thioalkyl
of 1 to 3 carbon atoms. Suitable carbocyclic aryl groups are
groups having one or two rings, at least one of which having
aromatic character and include carbocyclic aryl groups such as
phenyl and bicyclic carbocyclic aryl groups such as naphthyl.
As is apparent from the above formula, the compounds
encompassed therein may exist as racemic mixtures and as
individual stereoisomers. Especially preferred are
R-phenylpropyl α-phenethylamine derivatives which exhibit
enhanced activity in lowering serum ionized calcium.

       Preferred compounds include those where alk is
n-propylene. Also preferred are compounds where R_1 is

methyl. Also preferred are those compounds where R_2 and R_3 are optionally substituted phenyl.

Especially preferred compounds include those where R_2 is monosubstituted phenyl, more preferably meta-substituted. Especially preferred R_3 groups include unsubstituted or monosubstituted phenyl, especially ortho-substituted. Preferred substitutents for R_2 include halogen, haloalkyl, preferably trihalomethyl, and alkoxy, preferably methoxy. Preferred substituents for R_3 include halogen, preferably chlorine.

In a second related aspect, the invention fea tures a method for treating a patient having a disease or condition characterized by an abnormal $[Ca^{2+}]$ or $[Ca^{2+}]_i$ in one or more cells or in the blood or plasma or extracellular fluids. The method includes the step of administering to the patient a therapeutically effective amount of a molecule which either mimics the activity of extracellular Ca^{2+} by evoking an increase in $[Ca^{2+}]_i$ in a cell or blocks an increase in $[Ca^{2+}]_i$ elicited by extracellular Ca^{2+}.

By "abnormal" is meant that the patient, compared to the general population, has a different Ca^{2+} metabolism that is affected by one or more proteins (e.g., hormones) in the blood or extracellular body fluids, or other molecules which affect the level of extracellular and/or intracellular Ca^{2+}. Thus, the diseases include hyperparathyroidism, osteoporosis and other bone and mineral-related disorders, and the like (as described, e.g., in standard medical text books, such as "Harrison's Principles of Internal Medicine"). Such diseases are treated in this invention by molecules which mimic or block one or more of the effects of Ca^{2+} and

thereby directly or indirectly affect the levels of the
proteins or other molecules in the body of the patient.

By "therapeutically effective amount" is meant an
amount that relieves to some extent one or more symptoms of
the disease or condition in the patient. Additionally, by
"therapeutically effective amount" is meant an amount that
returns to normal, either partially or completely,
physiological or biochemical parameters associated with or
causative of the disease or condition. Generally, it is an
amount between about 1 nmole and 1 μmole of the molecule,
dependent on its EC_{50} and on the age, size, and disease
associated with the patient.

In preferred embodiments, the molecule has an EC_{50}
of less than or equal to 5 μM, and is not protamine; and
most preferably interacts at a Ca^{2+} receptor as a
calcimimetic or calcilytic. Most preferably the molecule is
chosen from one of those described above.

In other preferred embodiments, the patient has a
disease characterized by an abnormal level of one or more
components the level of which is regulated or affected by
activity of one or more Ca^{2+} receptors, and the molecule is
active on a Ca^{2+} receptor of a cell selected from the group
consisting of parathyroid cells, bone osteoclasts,
juxtaglomerular kidney cells, proximal tubule kidney cells,
distal tubule kidney cell, cell of the thick ascending limb of
Henle's loop and/or collecting duct, keratinocyte in the
epidermis, parafollicular cell in the thyroid (C-cells),
intestinal cell, trophoblast in the placenta, platelet,
vascular smooth muscle cell, cardiac atrial cell, gastrin and
glucagon secreting cells, kidney mesangial cell and mammary
cell.

- 22 -

In still other preferred embodiments, the mole cule reduces the level of parathyroid hormone in the serum of the patient, e.g., to that level present in a normal individual, or to a degree sufficient to cause a decrease in plasma Ca^{2+}; and the molecule is provided in an amount sufficient to have a therapeutically relevant effect on the patient.

In a third aspect, the invention features a method for diagnosis of a disease or condition in a patient by identifying the number and/or location (and/or functional integrity) of one or more Ca^{2+} receptors within the patient and comparing that number and/or location (and/or functional integrity) with that observed in normal patients as an indication of the presence of the disease or condition.

In preferred embodiments, the method is an immunoassay in which an antibody to a Ca^{2+} receptor is used to identify the number and/or location and/or functional integrity of the Ca^{2+} receptors, or the assay involves providing a labelled calcimimetic or calcilytic molecule which binds to a Ca^{2+} receptor; and the disease diagnosed is a cancer, e.g., an ectopic tumor of the parathyroid, or a condition characterized by an above normal level in the number of osteoclasts in bone or an increased level of activity of osteoclasts in bone.

In a fourth aspect, the invention features a method for identifying a molecule useful as a therapeutic molecule. The method includes screening a potentially useful molecule for either an ability to mimic the activity of extracellular Ca^{2+} in a cell, or to block an increase in $[Ca^{2+}]_i$ elicited by extracellular Ca^{2+}, and determining whether the molecule has an EC_{50} or IC_{50} of less than or equal to 5 μM.

- 23 -

In other aspects, the invention features a
recombinant Ca^{2+} receptor, a cell including a recombinant
Ca^{2+} receptor, purified nucleic acid encoding a Ca^{2+}
receptor, the biological activity and use of the molecule NPS
019, the novel compounds or compositions of matter of NPS 459,
NPS 467, and NPS 568 (see Fig. 36) and a method for
identifying a useful calcimimetic or calcilytic molecule by
identifying a molecule which mimics or blocks one or more
activities of Ca^{2+} at a first Ca^{2+} receptor but not at a
second Ca^{2+} receptor, e.g., by use of a recombinant Ca^{2+}
receptor.

In another aspect, a cell including a recombinant
Ca^{2+} receptor(s) from kidney is employed as a test system to
screen antibiotics (e.g. aminoglycoside antibiotics) for
activity at calcium receptors which produces renal toxicity in
humans.

By "recombinant" is meant to include any Ca^{2+}
receptor produced by recombinant DNA techniques such that it
is distinct from the naturally occurring Ca^{2+} receptor
either in its location, purity or structure. Generally, such
a receptor will be present in a cell in an amount different
from those normally observed in nature.

By "purified" is meant that the antibody or nucleic
acid is distinct from naturally occurring antibody or nucleic
acid, being separated from antibody or nucleic acid with which
it naturally occurs, e.g., in a vector system, such that it
can be used to express recombinant Ca^{2+} receptor.
Preferably, the antibody or nucleic acid is provided as a
homogeneous preparation by standard techniques.

Such cloned receptors can be expressed in a desired

cell, and isolated and crystallized to allow structure
determination. Such a structure will allow design of useful
molecules of this invention which can bind to the Ca^{2+}
receptor. In addition, equivalent such receptors can be
cloned using a first clone as a probe for clones in other
cell, cDNA or genomic libraries.

Antibodies to the cloned receptor can be isolated
and used as therapeutics in this invention, or as diagnostic
tools for determining Ca^{2+} receptor numbers and/or locations
and/or functional integrity to diagnose Ca^{2+}-related
diseases or conditions. Such antibodies can also be used _in
vivo_ by intravenous administration as calcimimetics or
calcilytics.

Thus, in general, the invention features
calcimimetic or calcilytic molecules able to act as either
selective agonists or antagonists respectively at a Ca^{2+}
receptor of one or more but not all cells chosen from the
group consisting of parathyroid cells, bone osteoclasts,
juxtaglomerular kidney cells, proximal tubule kidney cells,
distal tubule kidney cell, cell of the thick ascending limb of
Henle's loop and/or collecting duct, keratinocyte in the
epidermis, parafollicular cell in the thyroid (C-cells),
intestinal cell, trophoblast in the placenta, platelet,
vascular smooth muscle cell, cardiac atrial cell, gastrin and
glucagon secreting cells, kidney mesangial cell and mammary
cell. Such a composition may include any pharmaceutically
acceptable carrier known to those in the art to provide a
pharmaceutical composition.

The invention also features modulation of the number
of Ca^{2+} receptors in a patient by standard techniques, _e.g._,

- 25 -

antisense and related technologies (e.g., ribozymes), as a
therapeutic for a disease state.

This invention provides methods for identifying
molecules which affect the activity of a Ca^{2+} receptor using
assays, as defined below, to detect calcimimetics and/or
calcilytics. Further, molecules found to be effective to
reduce or enhance expression of Ca^{2+} receptor at a
transcriptional or translational level by use of the assays or
antibodies or other techniques described below can be defined
for therapeutic uses.

The foregoing summary has been largely in connection
with the preferred embodiment which relates generally to
calcium receptors. In a further aspect, the invention relates
to a novel superfamily of polypeptide receptor/sensor
molecules. The cDNA clone, BoPCaR 1, disclosed herein and
duly deposited, represents the first such clone described to
encode a receptor/sensor which is a member of this
superfamily. For purposes of the present invention,
receptor/sensors belonging to this superfamily are called
inorganic-ion receptors.

The novel superfamily of inorganic ion receptors
includes a variety of such molecules which are related to each
other by similarity of amino acid sequence, by structure
and/or by function. In total, these attributes also
distinguish members of this superfamily of receptors from all
receptors/sensors currently known in the art. Members of this
superfamily of receptors are primarily distinguished
functionally by their surprising ability to detect and respond
to changes in the levels of inorganic cations such as calcium,
magnesium, potassium, sodium, or hydrogen ions and the like,

and upon sensing such ions, to evoke changes in cellular
functions. Such changes may involve changes in second
messenger levels as occur for known G-protein coupled
receptors, or changes in ionic transmembrane ion flux or the
like. Other members of this superfamily also exist which
sense inorganic anions such as phosphate or chloride ions.
All such receptors are within the scope of the current
invention as described herein. Additionally, all isolated
naturally occurring or synthetic ligands for receptors/sensors
belonging to the superfamily of inorganic-ion receptors are
within the scope of the invention. In an additional aspect,
the invention features the therapeutic use of any such natural
or synthetic ligands for inorganic-ion receptors.

It is a further aspect of the present invention that
receptors belonging to the superfamily of inorganic-ion
receptors may be activated by stimuli other than ligand
binding. For example, some members of this superfamily of
receptors are activated by physical forces such as stretch
forces acting on membranes of cells expressing such
receptors. All such receptors are within the scope of the
present invention.

The inorganic-ion receptors can be used in the
manner as described above in connection with the preferred
receptor. Recombinant receptors expressed in a variety of
tissue types including human tissue types can be used to
screen (including high through-put screens) for drug discovery
in methods known to those skilled in the art. Ionmimetics and
ionlytics can be readily identified. The procedures can be
adapted for the individual function of the receptor. For
example, calcium activated chloride current may be employed

with an inorganic-ion receptor which forms a calcium channel
or which nutrilizes intracellular calcium. Ligand-gated ion
channels are known in the art. Inorganic-ion receptors that
couple to second messenger systems permit assays as described
above, including measurement of cyclic AMP and inositol
phosphates. Cells that have recombinant receptors coupled to
readily detectable agents also may be used such as G-protein
coupling of receptors to pigment dispersion in melanophores as
described in the literature. Thus, the invention fully
enables methods for discovering agents that are inorganic-ion
receptor mimetics or lytics, as well as such mimetics and
lytics themselves, including those preferably selected from
the mimetics and lytics described in detail above. Likewise,
the invention embraces diagnosis and treatment of
inorganic-ion receptor related diseases or conditions as
described above in connection with the preferred embodiment.
For example, diseases associated with elevated levels of an
inorganic-ion receptor can be diagnosed based upon assays for
such elevated levels. Therapeutic molecules can be identified
by screening for agents that mimic the activity of the
relevant native ion or that modify the effect or potency of
the relevant native ion. Tissue specific expression as well
as levels of expression can be evaluated and the like.

      Thus, in this aspect of the invention, a novel
superfamily of isolated inorganic-ion receptors is provided
and unique fragments thereof also are provided. Preferably,
the isolated receptors are human receptors, and most
preferably the isolated receptor is a calcium receptor
expressed in tissues or cells selected from the group
consisting of: parathyroid, vascular, kidney, epidermis,
thyroid, osteoclast, intestine, mammary, trophoblast,

- 28 -

platelet, gastrin secreting, glucagon secreting, cardiac, and
brain, and unique fragments thereof. The invention further
features polypeptide fragments of the foregoing inorganic-ion
receptors which have desirable activity. For example, the
fragment may include just a binding site, or a site which
binds to mimics, agonists or antagonists. Other useful
fragments include those that have only the external portion,
membrane-spanning portions, or intracellular portion of the
receptor. In addition, these fragments are useful for forming
chimeric receptors with fragments of other receptors, as
described in greater detail below. The invention also
features muteins or analogues and other derivatives of the
isolated receptors. Thus, the invention embraces not only
naturally occurring proteins, but derivatives thereof.

        The invention also features nucleotide sequences
encoding the foregoing inorganic-ion receptors and fragments
and derivatives thereof. Such nucleotide sequences may be
obtained through a variety of procedures, and the disclosure
of the present invention allows one of ordinary skill in the
art to obtain cDNA or genomic clones encoding such receptors.
For example, hybridization probes may be made based upon the
nucleotide sequence of BoPCaR 1. When genomic libraries for
cDNA libraries from any tissue are screened at low stringency
with such probes, hybridizing clones are obtained which encode
other members of the superfamily. Additionally, antibodies
can readily be prepared which bind to cloned or isolated
inorganic-ion receptors. Such antibodies may also be used to
isolate other receptors of the invention by expression-cloning
techniques known to those of ordinary skill in the art.
Targeted gene walking also may be employed to identify and

clone members of the superfamily of inorganic-ion receptors.
In addition, the clones may be obtained through expression
cloning procedures as described above. These procedures can
be adapted for the individual function of the inorganic-ion
receptor of interest. For example, calcium activated chloride
currents may also be employed for cloning of an inorganic-ion
receptor which forms a calcium channel or which mobilizes
intracellular calcium. Ligand-gated ion channels, as
discussed above, are known in the art. For example, a
serotonin gated ion channel has been cloned in this manner.
However, the receptors of the present invention are distinct
from other known ligand gated ion channels in their amino acid
sequences and in that they sense inorganic ions such as
calcium, magnesium, hydrogen ions, phosphate ions, and the
like.

It will also be appreciated by those skilled in the
art that each cloned receptor obtained in this manner provides
new such DNA or antibody probes which themselves are used to
identify still additional clones encoding inorganic-ion
receptors. Furthermore, it will be appreciated that having
obtained the sequence of more than one such receptor as
outlined above, information is available pertaining to
localized sequence conservation which is useful for obtaining
still additional clones encoding other members of the
superfamily. Such conserved sequences also may be derived
from an analysis of the overall structure of BoPCaR 1, as it
conventionally includes an extracellular domain, transmembrane
domain and intracellular domain.

Thus, isolated nucleic acids encoding inorganic-ion
receptors and unique fragments thereof are provided. The
preferred receptor is a calcium receptor that is expressed in

the tissues or cells selected from the group described above.
Most preferably, the nucleic acid encodes a human
inorganic-ion receptor or a unique fragment thereof. The
invention further provides isolation of the endogenous
regulatory elements controlling the expression of the
foregoing inorganic-ion receptors, and agents that are capable
of agonizing or antagonizing the activity of these regulatory
elements also may be identified.

The invention further provides recombinant cells
expressing the nucleic acids of the invention. In such cells,
the nucleic acid may be under the control of its genomic
regulatory elements, or may be under the control of exogenous
regulatory elements including an exogenous promoter. By
"exogenous" it is meant a promoter that is not normally
coupled in vivo transcriptionally to the coding sequence for
the inorganic-ion receptor.

Purified antibodies to the foregoing inorganic-ion
receptors are provided as well as antibodies to allosteric
sites of such receptors and idiotypes of such receptors.

Inorganic-ion receptor binding agents coupled to a
toxin also are provided. Such agents may be antibodies and,
as such, are immunotoxins. Such immunotoxins may be used for
example in killing a cell expressing an inorganic-ion receptor
in vitro or in vivo.

In addition, the invention provides transgenic,
nonhuman mammals containing a transgene encoding an
inorganic-ion receptor or a unique fragment thereof. Such
transgenes may be useful in affecting or altering the
expression of an inorganic-ion receptor or in inactivating the
expression of an inorganic-ion receptor through, for example,

- 31 -

homologous recombination. Other transgenes may be provided in such mammals, including those encoding antisense or a protein that is capable of altering the expression of a native inorganic-ion receptor gene. Such alteration may be up-regulation, down-regulation or complete inactivation.

Thus, methods are provided involving contacting a cell with an agent that binds to a cellular component for the purpose of affecting the expression of an inorganic-ion receptor. Such agents may bind to promoters, other regulatory agents acting on promoters, agents capable of binding to the receptor and nucleic acids encoding the receptor or a unique fragment thereof. Contact may be by extracellular administration, by providing a transgene encoding the agent or by any other suitable method depending upon the use to which the particular method is directed.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Description of the Preferred Embodiments

The drawings will first briefly be described.

Drawings

Fig. 1 depicts representative molecules useful in the invention.

Fig. 2 is a graphical representation showing increases in $[Ca^{2+}]_i$ induced by extracellular Ca^{2+} in quin-2- or fura-2-loaded bovine parathyroid cells. The initial $[Ca^{2+}]$ was 0.5 mM (using $CaCl_2$) and, at each of the arrows, was increased in 0.5 mM increments.

Fig. 3 is a graphical representation showing mobilization of $[Ca^{2+}]_i$ in bovine parathyroid cells. The

initial $[Ca^{2+}]$ was 0.5 mM and was decreased to < 1 μM by
the addition of EGTA as indicated. (a) Extracellular Mg^{2+}
(8 mM, final) elicits an increase in $[Ca^{2+}]_i$ in the
absence of extracellular Ca^{2+}. (b) Pretreatment with
ionomycin (1 μM) blocks the response to Mg^{2+}. (c)
Pretreatment with 5 μM molecule 1799 (a mitochondrial
uncoupler) is without effect on the response to Mg^{2+}.

Fig. 4 is a graphical representation showing
preferential inhibitory effects of a low concentration of
Gd^{3+} on steady-state increases in $[Ca^{2+}]_i$ and that a
high concentration of Gd^{3+} elicits a transient increase in
$[Ca]_i$ in bovine parathyroid cells. Top panel: Control.
Initial concentration of extracellular Ca^{2+} was 0.5 mM and
was increased by 0.5 mM at each of the arrowheads. Middle
panel: Gd^{3+} (5 μM) blocks steady-state but not transient
increases in $[Ca^{2+}]_i$ elicited by extracellular Ca^{2+}.
Lower panel: Gd^{3+} (50 μM) elicits a transient increase in
$[Ca^{2+}]_i$ and abolishes both transient and sustained
responses to extracellular Ca^{2+}. In the middle and lower
panels, just enough EGTA was added to chelate preferentially
Gd^{3+}: the block of Ca^{2+} influx is removed and $[Ca^{2+}]_i$
rises promptly.

Fig. 5 is a graphical representation showing that
the effects of PMA on $[Ca^{2+}]_i$, IP_3 formation, and PTH
secretion are overcome by increasing concentrations of
extracellular Ca^{2+} in bovine parathyroid cells. For each
variable, there is a shift to the right in the
concentration-response curve for extracellular Ca^{2+}. Note
also that the concentration- response curves vary sigmoidally
as $[Ca^{2+}]$ increases linearly.

- 33 -

Fig. 6 is a graphical representation showing that increases in $[Ca^{2+}]_i$ elicited by spermine are progressively depressed by increasing $[Ca^{2+}]$ in bovine parathyroid cells. Spermine (200 µM) was added at the time shown by arrowheads. In this and all subsequent figures, the numbers accompanying the traces are $[Ca^{2+}]_i$ in nM.

Fig. 7 is a graphical representation showing that spermine mobilizes intracellular Ca^{2+} in bovine parathyroid cells. EGTA was added to reduce $[Ca^{2+}]$ to <1 àM before the addition·of spermine (200 µM) as indicated (left trace). Pretreatment with ionomycin (1 µM) blocks the response to spermine (right trace).

Figs. 8A and B are graphical representations showing that spermine increases $[Ca^{2+}]_i$ and inhibits PTH secretion in bovine parathyroid cells similarly to extracellular Ca^{2+}. The data points for the spermine dose-concentration response curves are the means of two experiments.

Fig. 9 is a graphical representation showing the contrasting effects of PMA on responses to extracellular Ca^{2+} and on responses to ATPγS in bovine parathyroid cells. Left panel: The concentration- response curve for extracellular Ca^{2+}-induced inhibition of cyclic AMP formation is shifted to the right by PMA (100 nM). Middle panel: PMA does not affect the ability of ATPγS to increases $[Ca^{2+}]_i$. Note also that the concentration-response curve to ATPγS shows classical sigmoidal behavior as a function of the log concentration, in contrast to extracellular divalent cations.

Fig. 10 is a graphical representation showing mobilization of intracellular Ca^{2+} in human parathyroid

- 34 -

cells evoked by extracellular Mg^{2+}. Cells were obtained
from an adenoma and bathed in buffer containing 0.5 mM
extracellular Ca^{2+}. (a) Transient and sustained increases in
$[Ca^{2+}]_i$ elicited by extracellular Mg^{2+} (10 mM, final)
shows that sustained increases are not affected by nimodipine
(1 μM) but are depressed by La^{3+} (1 μM) and return
promptly when La^{3+} is selectively chelated by a low
concentration of EGTA. (b) La^{3+} (1 μM) blocks the
sustained but not the transient increase in $[Ca^{2+}]_i$
elicited by extracellular Mg^{2+}. (c) Cytosolic Ca^{2+}
transients elicited by extracellular Mg^{2+} persist in the
absence of extracellular Ca^{2+}.

Fig. 11 is a graphical representation showing
mobilization of intracellular Ca^{2+} evoked by neomycin or
protamine in bovine parathyroid cells. In all traces, the
initial $[Ca^{2+}]$ and $[Mg^{2+}]$ was 0.5 and 1 mM, respectively.
In trace (a) and (b), the Ca^{2+} and Mg^{2+} concentrations
were increased to 2 and 8 mM, from 0.5 and 1 mM respectively.
In the other traces, (c) through (i), neomycin B (30 μM) or
protamine (1 μg/ml) were added as indicated. La^{3+}
(1 μM) EGTA (1 mM), or ionomycin (100 nM) were added as
indicated. Each trace is representative of the pattern seen
in 5 or more trials using at least 3 different cell
preparations. Bar = 1 min.

Fig. 12 is a graphical representation showing that
neomycin B blocks transient but does not block steady-state
increases in $[Ca^{2+}]_i$ elicited by extracellular Ca^{2+} in
bovine parathyroid cells. Left control: $[Ca^{2+}]$ was
initially 0.5 mM and was increased in 0.5 mM increments at
each of the open arrowheads before the addition of neomycin B

- 35 -

(30 µM). Right: Neomycin B (30 µM) was added before
$[Ca^{2+}]$. Bar = 1 min.

Fig. 13 is a graphical representation showing that
neomycin B or protamine inhibit PTH secretion at
concentrations which evoked increases in $[Ca^{2+}]_i$ in bovine
parathyroid cells. Cells were incubated with the indicated
concentrations of organic polycation for 30 min. in the
presence of 0.5 mM extracellular Ca^{2+}. Open symbols:
control responses for PTH secretion in the presence of 0.5
(circles) or 2 mM (diamonds) extracellular Ca^{2+}. Values for
$[Ca^{2+}]_i$ are diamond symbols. Bovine cells were used in
the experiments with protamine and human (adenoma) parathyroid
cells were used in the experiments with neomycin B. Each
point is the mean ± SEM of 3 experiments.

Fig. 14 is a graphical representation showing the
preferential inhibitory effects of PMA on cytosolic Ca^{2+}
transients elicited by spermine in bovine parathyroid cells.
Initial $[Ca^{2+}]$ was 0.5 mM; spermine (200 µM) or ATP (50
µM) were added as indicated. Bar = 1 min.

Fig. 15 is a graphical representation showing that
PMA shifts to the right the concentration-response curves for
extracellular Ca^{2+}- and neomycin B-induced increases in
$[Ca^{2+}]_i$ in bovine parathyroid cells. Cells were
pretreated with PMA for 1 min. before increasing $[Ca^{2+}]$ or
before adding neomycin B as indicated. Each point is the mean
± SEM of 3 to 5 experiments.

Fig. 16 is a graphical representation showing that
PMA shifts to the right the concentration-response curves for
extracellular Ca^{2+}- and spermine-induced inhibition of PTH
secretion in bovine parathyroid cells. Cells were incubated

- 36 -

with the indicated $[Ca^{2+}]$ and spermine for 30 min. in the presence (closed circles) or absence (open circles) of 100 nM PMA. Each point is the mean $\pm$ SEM of 3 experiments.

Fig. 17 is a graphical representation showing that protamine increases the formation of inositol phosphates in bovine parathyroid cells. Parathyroid cells were incubated overnight in culture media containing 4 μCi/ml ^{3}H-myo-inositol, washed, and incubated with the indicated concentration of protamine at 37°. After 30 sec. the reaction was terminated by the addition of $CHCl_3$:MeOH:HCl and IP_1 (circles) and IP_3 (triangles) separated by anion exchange chromatography. Each point is the mean of 2 experiments, each performed in triplicate.

Fig. 18 is a graphical representation showing that PMA depresses the formation of IP_1 evoked by extracellular Ca^{2+} or spermine in bovine parathyroid cells. ^{3}H-myo-inositol- labeled cells were exposed to the indicated $[Ca^{2+}]$ or spermine for 30 sec. before terminating the reaction and determining IP_1 by anion exchange chromatography. Hatched columns: Cells were pretreated with PMA (100 nM) for 5 min. before increasing $[Ca^{2+}]$ or adding spermine. Each value is the mean of 2 experiments, each performed in triplicate.

Fig. 19 is a graphical representation showing transient and sustained increases in $[Ca^{2+}]_i$ elicited by neomycin B in human (adenoma) parathyroid cells. $[Ca^{2+}]$ was 0.5 mM. (a) The sustained increase in $[Ca^{2+}]_i$ elicited by neomycin B (10 μM) was depressed by La^{3+}. (b) The transient increase in $[Ca^{2+}]_i$ evoked by neomycin B was unaffected by La^{3+}. (c) Transient increases in $[Ca^{2+}]_i$

- 37 -

persisted in the absence of extracellular Ca^{2+}.

Fig. 20 is a graphical representation showing that neomycin B evokes oscillating increases the Cl^- current in Xenopus oocytes expressing the Ca^{2+} receptor. Upper trace from an oocyte three days after injection with human (hyperplastic) parathyroid cell poly$(A)^+$-mRNA. Lower trace from an oocyte injected with water. Neomycin B failed to elicit a response in five water-injected oocytes and carbachol elicited a response in one, which is shown. In both traces, the holding potential was -76 mV.

Fig. 21 is a graphical representation showing that neomycin B fails to affect basal or evoked increases in C-cells. Control, left trace: Fura-2- loaded rMTC 6-23 cells were initially bathed in buffer containing 1 mM Ca^{2+} before increasing $[Ca^{2+}]$ to 3 mM. Right trace: pretreatment with 5 mM neomycin B.

Fig. 22 is a graphical representation showing that extracellular Ca^{2+} evokes increases in $[Ca^{2+}]_i$ in rat osteoclasts. Microfluorimetric recording in a single rat osteoclast loaded with indo-1 and superfused for the indicated times (bars) with buffer containing the indicated $[Ca^{2+}]$. Normal buffer, superfused between the bars, contained 1 mM Ca^{2+}.

Fig. 23 is a graphical representation showing that spermine or neomycin B fail to evoke increases in $[Ca^{2+}]_i$ in rat osteoclasts. An indo-1-loaded osteoclast was superfused with the indicated concentration of spermine or neomycin B (open bars) alone or together with 20 mM Ca^{2+} (solid bars).

- 38 -

Fig. 24 is a graphical representation showing the differential effects of argiotoxin (shown as argiopine in the figure, structures also shown in Fig. 1) 659 and argiotoxin 636 on $[Ca^{2+}]_i$ in bovine parathyroid cells. The initial $[Ca^{2+}]$ was 0.5 mM and was increased to 1.5 mM where indicated (right trace)N Where indicated, argiotoxin 659 (300 µM) or argiotoxin 636 (400 µM) was added.

Fig. 25 is a graphical representation showing that extracellular Mg^{2+} or Gd^{3+} evoke oscillatory increases in Cl^- current in <u>Xenopus</u> oocytes injected with bovine parathyroid cell poly(A)$^+$-mRNA. In trace (a), the concentration of extracellular Ca^{2+} was < 1 µM and in trace (b), 0.7 mM. Trace (c) shows that extracellular Mg^{2+} fails to elicit a response in an oocyte injected only with the mRNA for the substance K receptor, although superfusion with substance K evokes a response. Holding potential was -70 to -80 mV.

Fig. 26 is a graphical representation showing that extracellular Ca^{2+} elicits oscillatory increases in Cl^- current in <u>Xenopus</u> oocytes injected with human (hyperplastic) parathyroid tissue poly(A)$^+$-mRNA. The oocyte was tested for responsivity to extracellular Ca^{2+} three days after injection of 50 ng poly(A)$^+$-mRNA. Holding potential was -80 mV.

Fig. 27 is a graphical representation showing the mobilization of intracellular Ca^{2+} in bovine parathyroid cells elicited by budmunchiamine. Budmunchiamine (300 µM, structure also shown) was added where indicated.

Fig. 28 is a graphical representation showing that the ability to mobilize intracellular Ca^{2+} in parathyroid

cells is stereospecific. Bovine parathyroid cells loaded with
fura-2 were initially suspended in buffer containing 0.5 mM
extracellular Ca^{2+} before the addition of the indicated
concentration of each molecule.

Fig. 29 is a graphical representation showing
effects of La^{3+} on $[Ca^{2+}]_i$ in osteoclasts. A
representative trace from a single rat osteoclast loaded with
indo-1 is shown. At low concentrations, La^{3+} partially
blocks increases in $[Ca^{2+}]_i$, elicited by extracellular
Ca^{2+}.

Figs. 30A and B are graphical representations
showing the mobilization of intracellular Ca^{2+} elicited by
extracellular Mn^{2+} in rat osteoclasts. Extracellular Mn^{2+}
evokes concentration-dependent increases in $[Ca^{2+}]_i$ (Fig.
30A) that persist in the absence of extracellular Ca^{2+} (Fig.
30B).

Figs. 31A and 31B are graphical representations
showing mobilization of $[Ca^{2+}]_i$ in rat osteoclasts
elicited by a molecule termed NPS 449 (see Fig. 38). Isolated
rat osteoclasts loaded with indo-1 were superfused with the
indicated concentrations of NPS 449 in the presence (Fig. 31A)
or absence (Fig. 31B) of 1 mM extracellular $CaCl_2$.

Fig. 32 is a graphical representation showing the
mobilization of intracellular Ca^{2+} in C-cells evoked by NPS
019 (see Fig. 1). rMTC 6-23 cells were loaded with fura-2 and
bathed in buffer containing 0.5 mM $[Ca^{2+}]$. Where indicated,
NPS 019 was added to a final concentration of 10 μM.
Representative traces show that the transient increase in
$[Ca^{2+}]_i$ elicited by NPS 019 is refractory to inhibition by
La^{3+} (middle trace) and persists in the absence of

extracellular Ca^{2+} (right trace).

Fig. 33 is a graphical representation showing that NPS 456 (Fig. 36) evokes oscillatory increases in Cl$^-$ current in Xenopus oocytes which have been injected with bovine parathyroid cell poly(A)$^+$-mRNA.

Fig. 34 is a graphical representation showing that extracellular Ca^{2+} evokes oscillatory increases in Cl$^-$ current in Xenopus oocytes which have been injected with human osteoclast mRNA. The oocyte was tested for responsivity to extracellular Ca^{2+} three days after injection of 50 ng of total poly(A)$^+$ mRNA.

Fig. 35 is a graphical representation showing that the parathyroid cell Ca^{2+} receptor is encoded by mRNA in a size range of 2.5-3.5 kb. Bovine parathyroid cell poly(A)$^+$ -mRNA was size fractionated on glycerol gradients and pooled into ten fractions. Each fraction was injected (50 ng/fraction) separately into Xenopus oocytes. After three days, the oocytes were examined for their ability to respond to neomycin B (10mM) with oscillatory increases in the Cl$^-$ current.

Fig. 36 shows the chemical structures of molecules derived from diphenylpropyl-α-phenethylamine illustrating a family of molecules which were prepared and screened to find the useful molecules of the invention.

Fig. 37 is a graphical representation showing that NPS 021 is a calcilytic compound that blocks the effects of extracellular Ca^{2+} on [Ca^{2+}]$_i$ in bovine parathyroid cells. Cells were initially bathed in buffer containing 0.5 mM CaCl$_2$ and, where indicated, the [Ca^{2+}] was increased to a final of 2mM (left trace). The addition of NPS 021

- 41 -

(200 µM) caused no change in $[Ca^{2+}]_i$ but inhibited the increase in $[Ca^{2+}]_i$ elicited by extracellular Ca^{2+} (right trace).

Fig. 38 is a graph showing <u>in vivo</u> Ca^{2+} response to NPS R,S-467.

Fig. 39 is a graph showing <u>in vivo</u> PTH response to NPS R,S-467.

Fig. 40 is a graph showing <u>in vivo</u> serum Ca^{2+} response to 25 mg/kg NPS R,S-467.

Fig. 41 is a graph showing the <u>in vivo</u> response of $[Ca^{2+}]_i$ in bovine parathyroid cells to different enantiomers of NPS 467.

Fig. 42 is a graph showing the <u>in vivo</u> response of serum Ca^{2+} in rats to different enantiomers of NPS 467.

Fig. 43a depicts a reaction scheme for the preparation of fendiline or fendiline analogs or derivatives depicted in Figure 36. Fig. 43b depicts a reaction scheme for the synthesis of NPS 467.

Fig. 44 depicts a dose-response curve showing that NPS 467 lowers serum ionized calcium in rats when administered orally.

Fig. 45 is a restriction map of the plasmid containing BoPCaR 1, deposited with the ATCC under accession number ATCC 75416.

Fig. 46 is a restriction map of BoPCaR 1.

Fig. 47 is a nucleotide sequence corresponding (about 90% accurate) to the 2.2 Kbfragment of BoPCaR 1 sequenced.

<u>Calcimimetic and Calcilytic Molecules</u>

Calcimimetic and calcilytic molecules useful in the invention are generally described above. These molecules

- 42 -

can be readily identified using screening procedures to
define molecules which mimic or antagonize the activity of
Ca^{2+} at Ca^{2+} receptors. Examples of such procedures are
provided below. These examples are not limiting in the
invention but merely illustrate methods which are readily
used or adapted by those skilled in the art.

Generally, calcimimetic and calcilytic molecules are
identified by screening molecules which are modelled after
those described below (called lead molecules). As can be
seen below there are several specific calcimimetics and
calcilytics useful at various Ca^{2+} receptors. Derivative
molecules are readily designed by standard procedures and
tested in one of many protocols known to those skilled in
the art. Many molecules may be screened easily to identify
the most useful in this invention.

Organic cationic molecules which mimic or antagonize
the actions of Ca^{2+} in other systems contain the requisite
structure for activity on a Ca^{2+} receptor. Rational
design of other useful molecules involves the study of a
molecule known to be calcimimetic or calcilytic and then
modifying the structure of the known molecule. For example,
polyamines are potentially calcimimetic since spermine
mimics the action of Ca^{2+} in several in vitro systems.
Results show that spermine does indeed cause changes in
$[Ca^{2+}]_i$ and PTH secretion reminiscent of those elicited
by extracellular di- and trivalent cations (see below).
Conversely, Ga^{3+} antagonizes the effects of Gd^{3+} on the
bovine parathyroid calcium receptor(s). The experiments
outlined below are therefore aimed at demonstrating that
this phenomenology, obtained with spermine, involves the

– 43 –

same mechanisms used by extracellular Ca^{2+}. To do this, the effects of spermine on a variety of physiological and biochemical parameters which characterize activation of the Ca^{2+} receptor were assessed. Those molecules having similar effects are useful in this invention and can be discovered by selecting or making molecules having a structure similar to spermine. Once another useful molecule is discovered this selection process can be readily repeated.

For clarity, below is provided a specific series of screening protocols to identify such useful molecules which are active at a parathyroid cell Ca^{2+} receptor, or which act as agonists or antagonists of the cellular response to changes in $[Ca^{2+}]$. Equivalent assays can be used for molecules active at other Ca^{2+} receptors or other inorganic-ion receptors, or which otherwise mimic or antagonize cellular functions regulated by $[Ca^{2+}]$ or other ions. These assays exemplify the procedures which are useful to find molecules, including calcimimetic molecules, of this invention. Equivalent procedures can be used to find lytic molecules, including calcilytic molecules, by screening for those molecules most antagonistic to the actions of the ion, including extracellular Ca^{2+}. In vitro assays can be used to characterize the selectivity, saturability, and reversibility of these mimetics and lytics by standard techniques.

Screening Procedure

Generally, bovine parathyroid cells loaded with fura-2 are initially suspended in buffer containing 0.5 mM $CaCl_2$. The test substance is added to the cuvette in a

small volume (5-15 µl) and any change in the fluorescence
signal noted. Cumulative increases in the concentration of
the test substance are made in the cuvette until some
predetermined concentration is achieved or changes in
fluorescence noted. If no changes in fluorescence are
noted, the molecule is considered inactive and no further
testing is performed. In initial studies, e.g., with
polyamine-type molecules, molecules were tested at
concentrations as high as 5 or 10 mM. As more potent
molecules are now known (see below), the ceiling
concentration is lowered. For example, newer molecules are
tested at concentrations up to 500 µM or less. If no
changes in fluorescence are noted at this concentration, the
molecule can be considered inactive.

Molecules causing increases in $[Ca^{2+}]_i$ are
subjected to additional testing. The two essential
characteristics of the molecule important for its
consideration as a calcimimetic molecule are the
mobilization of intracellular Ca^{2+} and sensitivity to PKC
activators. Molecules causing the mobilization of
intracellular Ca^{2+} in a PMA-sensitive manner have
invariably been found to be calcimimetic molecules and to
inhibit PTH secretion. Additional testing can, if needed,
be performed to solidify this belief. Typically, all the
various tests for calcimimetic or calcilytic activity (see
above) are not performed. Rather, if a molecule causes the
mobilization of intracellular Ca^{2+} in a PMA-sensitive
manner, it is advanced to screening on human parathyroid
cells. For example, measurements of $[Ca^{2+}]_i$ are
performed to determine the EC_{50}, and to measure the

ability of the molecule to inhibit PTH secretion in human
parathyroid cells which have been obtained from patients
undergoing surgery for primary or secondary
hyperparathyroidism. The lower the EC_{50} or IC_{50} the
more potent the molecule as a calcimimetic or calcilytic.

Measuring $[Ca^{2+}]_i$ with fura-2 provides a very rapid
means of screening new organic molecules for activity. In a
single afternoon, 10-15 molecules can be examined and their
ability to mobilize intracellular Ca^{2+} (or not) assessed.
The sensitivity of any observed increase in $[Ca^{2+}]_i$ to
depression by PMA can also be assessed. Moreover, a single
cell preparation can provide data on $[Ca^{2+}]_i$, cyclic AMP
levels, IP_3 and PTH secretion. A typical procedure is to
load cells with fura-2 and then divide the cell suspension
in two; most of the cells are used for measurement of
$[Ca^{2+}]_i$ and the remainder are incubated with molecules
to assess their effects on cyclic AMP and PTH secretion.
Because of the sensitivity of the radioimmunoassays for
cyclic AMP and PTH, both variables can be determined in a
single incubation tube containing 0.3 ml cell suspension
(about 500,000 cells). Measurements of inositol phosphates
are a time-consuming aspect of the screening. However, ion-
exchange columns eluted with chloride (rather than formate)
provide a very rapid means of screening for IP_3 formation
since rotary evaporation (which takes around 30 hrs) is not
required. This method allows processing of nearly 100
samples in a single afternoon. Those molecules that prove
interesting, as assessed by measurements of $[Ca^{2+}]_i$,
cyclic AMP, IP_3, and PTH are then subjected to a more
rigorous analysis by examining formation of various inositol

- 46 -

phosphates and assessing their isomeric form by HPLC.

Interesting molecules detected in these protocols are then assessed for specificity, e.q., by examining their effects on $[Ca^{2+}]_i$ in calcitonin-secreting C-cells using, e.q., the rat MTC 6-23 cell line.

The following is illustrative of methods useful in these screening procedures. Examples of typical results for various test calcimimetic or calcilytic molecules are provided in Figs. 2-34.

Parathyroid Cell Preparation

Parathyroid glands were obtained from freshly slaughtered calves (12-15 weeks old) at a local abattoir and transported to the laboratory in ice-cold parathyroid cell buffer (PCB) which contains (mM): NaCl, 126; KCl, 4; $MgCl_2$, 1; Na-HEPES, 20; pH 7.4; glucose, 5.6, and variable amounts of $CaCl_2$, e.q., 1.25 mM. Human parathyroid glands, obtained from patients undergoing surgical removal of parathyroid tissue for primary or uremic hyperparathyroidism (HPT), were treated similarly to bovine tissue. Glands were trimmed of excess fat and connective tissue and then minced with a fine scissors into approximate cubes of 2-3 mm. Dissociated parathyroid cells were prepared by collagenase digestion. Dissociated cells were then purified by centrifugation in Percoll buffer. The resultant parathyroid cell preparation was essentially devoid of red blood cells, adipocytes, and capillary tissue as assessed by phase contrast microscopy and Sudan black B staining. Dissociated and purified parathyroid cells were present as small clusters containing 5 to 20 cells. Cellular viability, as indexed by exclusion of trypan blue

- 47 -

or ethidium bromide, was routinely 95%.

Although cells can be used for experimental
purposes at this point, physiological responses
(suppressibility of PTH secretion and resting levels of
$[Ca^{2+}]_i$) are better after culturing the cells
overnight. Primary culture also has the advantage that
cells can be labeled with isotopes to near isotopic
equilibrium, as is necessary for studies involving
measurements of inositol phosphate metabolism (see below).
After purification on Percoll gradients, cells were washed
several times in a 1:1 mixture of Ham's F12-Dulbecco's
modified Eagle's medium (GIBCO) supplemented with 50 μg/ml
streptomycin, 100 U/ml penicillin, 5 μg/ml gentamicin and
ITS^+. ITS^+ is a premixed solution containing insulin,
transferrin, selenium, and bovine serum albumin
(BSA)-linolenic acid (Collaborative Research, Bedford, MA).
The cells were then transferred to plastic flasks (75 or 150
cm^2; Falcon) and incubated at 37°C in a humid atmosphere
of 5% CO_2. No serum is added to these overnight cultures,
since its presence allows the cells to attach to the
plastic, undergo proliferation, and dedifferentiate. Cells
cultured under the above conditions were readily removed
from the flasks by decanting, and show the same viability as
freshly prepared cells.

Measurement of Cytosolic Ca^{2+}

Purified parathyroid cells were resuspended in
1.25 mM $CaCl_2$-2% BSA-PCB containing 1 μM
fura-2-acetoxymethylester and incubated at 37°C for 20 min.
The cells were then pelleted, resuspended in the same buffer
lacking the ester, and incubated a further 15 min at 37°C.

- 48 -

The cells were subsequently washed twice with PCB containing
0.5 mM $CaCl_2$ and 0.5% BSA and maintained at room
temperature (about 20°C). Immediately before use, the cells
were diluted five-fold with prewarmed 0.5 mM $CaCl_2$-PCB to
obtain a final BSA concentration of 0.1%. The concentration
of cells in the cuvette used for fluorescence recording was
1-2 x 10^6/ml.

The fluorescence of indicator-loaded cells was
measured at 37°C in a spectrofluorimeter (Biomedical
Instrumentation Group, University of Pennsylvania,
Philadelphia, PA) equipped with a thermostated cuvette
holder and magnetic stirrer using excitation and emission
wavelengths of 340 and 510 nm, respectively. This
fluorescence indicates the level of cytosolic Ca^{2+}.
Fluorescence signals were calibrated using digitonin
(50 µg/ml, final) to obtain maximum fluorescence (F_{max}),
and EGTA (10 mM, pH 8.3, final) to obtain minimal
fluorescence (F_{min}), and a dissociation constant of 224
nM. Leakage of dye is dependent on temperature and most
occurs within the first 2 min after warming the cells in the
cuvette; dye leakage increases only very slowly thereafter.
To correct the calibration for dye leakage, cells were
placed in the cuvette and stirred at 37°C for 2-3 min. The
cell suspension was then removed, the cells pelleted, and
the supernatant returned to a clean cuvette. The
supernatant was then treated with digitonin and EGTA as
above to obtain as estimate of dye leakage, which is
typically 10-15% of the total Ca^{2+}-dependent fluorescent
signal. This estimate was subtracted from the apparent
F_{min}.

- 49 -

Measurement of PTH Secretion

In most experiments, cells loaded with fura-2 were used in studies of PTH secretion. Loading parathyroid cells with fura-2 does not change their secretory response to extracellular Ca^{2+}. Cells were suspended in PCB containing 0.5 mM $CaCl_2$ and 0.1% BSA. Incubations were performed in plastic tubes (Falcon 2058) containing 0.3 ml of the cell suspension with or without small volumes of $CaCl_2$ and/or organic polycations. After incubation at 37°C for various times (typically 30 min), the tubes were placed on ice and the cells pelleted at 2°C. Samples of the supernatant were brought to pH 4.5 with acetic acid and stored at -70°C. This protocol was used for both bovine and human parathyroid cells.

For bovine cells, the amount of PTH in sample supernatants was determined by a homologous radioimmunoassay using GW-1 antibody or its equivalent at a final dilution of 1/45,000. ^{125}I-PTH (65-84; INCSTAR, Stillwater, MN) was used as tracer and fractions separated by dextran-activated charcoal. Counting of samples and data reduction were performed on a Packard Cobra 5005 gamma counter.

For human cells, a commercially available radioimmunoassay kit (INS-PTH; Nichols Institute, Los Angeles, CA) which recognizes intact and N-terminal human PTH was used because GW-1 antibody recognizes human PTH poorly.

Measurement of cyclic AMP

Cells were incubated as above for PTH secretion studies and at the end of incubation, a 0.15 ml sample was taken and transferred to 0.85 ml hot (70°C) water and heated

at this temperature for 5-10 min. The tubes were
subsequently frozen and thawed several times and the
cellular debris sedimented by centrifugation. Portions of
the supernatant were acetylated and cyclic AMP
concentrations determined by radioimmunoassay.

Measurement of Inositol Phosphate Formation

Membrane phospholipids were labeled by incubating
parathyroid cells with 4 µCi/ml ^{3}H-myo-inositol for
20-24 hrs. Cells were then washed and resuspended in PCB
containing 0.5 mM $CaCl_2$ and 0.1% BSA. Incubations were
performed in microfuge tubes in the absence or presence of
various concentrations of organic polycation for different
times. Reactions were terminated by the addition of 1 ml
chloroform/methanol/12 N HCl (200:100:1; v/v/v). Phytic
acid hydrolysate (200 µl; 25 µg phosphate/tube) water
was then added. The tubes were centrifuged and 600 µl of
the aqueous phase was diluted into 10 ml water.

Inositol phosphates were separated by ion-
exchange chromatography using AG1-X8 in either the chloride-
or formate-form. When only IP_3 levels were to be
determined, the chloride-form was used, whereas the formate
form was used to resolve the major inositol phosphates
(IP_3, IP_2, and IP_1). For determination of just IP_3,
the diluted sample was applied to the chloride-form column
and the column washed with 10 ml 30 mM HCl followed by 6 ml
90 mM HCl and the IP_3 eluted with 3 ml 500 mM HCl. The
last eluate was diluted and counted. For determination of
all major inositol phosphates, the diluted sample was
applied to the formate-form column and IP_1, IP_2, and
IP_3 eluted sequentially by increasing concentrations of

- 51 -

formate buffer. The eluted samples from the formate columns were rotary evaporated, the residues brought up in cocktail, and counted.

The isomeric forms of IP_3 were evaluated by HPLC. The reactions were terminated by the addition of 1 ml 0.45 M perchloric acid and stored on ice for 10 min. Following centrifugation, the supernatant was adjusted to pH 7-8 with $NaHCO_3$. The extract was then applied to a Partisil SAX anion-exchange column and eluted with a linear gradient of ammonium formate. The various fractions were then desalted with Dowex followed by rotary evaporation prior to liquid scintillation counting in a Packard Tri-carb 1500 LSC.

For all inositol phosphate separation methods, appropriate controls using authentic standards were used to determine if organic polycations interfered with the separation. If so, the samples were treated with cation-exchange resin to remove the offending molecule prior to separation of inositol phosphates.

Measurement of Cytosolic Ca^{2+} in C-cells

Neoplastic C-cells derived from a rat medullary thyroid carcinoma (rMTC 6-23) obtained from American Type Culture Collection (ATCC No. 1607) were cultured as monolayers in Dulbecco's Modified Eagle's medium (DMEM) plus 15% horse serum in the absence of antibiotics. For measurements of $[Ca^{2+}]_i$, the cells were harvested with 0.02% EDTA/0.05% trypsin, washed twice with PCB containing 1.25 mM $CaCl_2$ and 0.5% BSA, and loaded with fura-2 as described above for parathyroid cells. Measurements of $[Ca^{2+}]_i$ were performed as described above with

- 52 -

appropriate corrections for dye leakage.

Measurement of $[Ca^{2+}]_i$ in Rat Osteoclasts

Osteoclasts were obtained from 1-2 day old
Sprague-Dawley rats using aseptic conditions. The rat pups
were sacrificed by decapitation, the hind legs removed, and
the femora rapidly freed of soft tissue and placed in
prewarmed F-12/DMEM media (DMEM containing 10% fetal calf
serum and antibiotics (penicillin-streptomycin-gentamicin;
100 U/ml-100 µg/ml-100 µg/ml)). The bones from two pups
were cut lengthwise and placed in 1 ml culture medium. Bone
cells were obtained by gentle trituration of the bone
fragments with a plastic pipet and diluted with culture
medium. The bone fragments were allowed to settle and equal
portions (about 1 ml) of the medium transferred to a 6-well
culture plate containing 25 mm glass coverslips. The cells
were allowed to settle for 1 hr at 37°C in a humidified 5%
CO_2-air atmosphere. The coverslips were then washed 3
times with fresh media to remove nonadherent cells.
Measurements of $[Ca^{2+}]_i$ in osteoclasts were performed
within 6-8 hrs of removing nonadherent cells.

Cells attached to the coverslip were loaded with
indo-1 by incubation with 5 µM indo-1 acetoxymethylester /
0.01% Pluronic F28 for 30 min at 37°C in F-12/DMEM lacking
serum and containing instead 0.5% BSA. The coverslips were
subsequently washed and incubated an additional 15 min at
37°C in F-12/DMEM lacking ester before being transferred
to a superfusion chamber mounted on the stage of a Nikon
Diaphot inverted microscope equipped for microfluorimetry.
Osteoclasts were easily identified by their large size and
presence of multiple nuclei. The cells were superfused with

buffer (typically PCB containing 0.1% BSA and 1 mM Ca^{2+})
at 1 ml/min with or without test substance. The
fluorescence emitted by excitation at 340 nm was directed
through the video port of the microscope onto a 440 nm
dichroic mirror and fluorescence intensity at 495 and 405 nm
collected by photomultiplier tubes. The outputs from the
photomultiplier tubes were amplified, digitized, and stored
in an 80386 PC. Ratios of fluorescence intensity were used
to estimate $[Ca^{2+}]_i$.

Oocyte Expression

In additional studies, _Xenopus_ oocytes injected
with mRNA from bovine or human parathyroid cells were used
in screening protocols, and Cl^- current measured as an
indirect means of monitoring increases in $[Ca^{2+}]_i$. The
following is an example to test the effect of neomycin.

Oocytes were injected with $poly(A)^+$-enriched
mRNA from human parathyroid tissue (hyperplastic glands from
a case of secondary HPT). After 3 days, the oocytes were
tested for their response to neomycin. Neomycin B evoked
oscillatory increases in the Cl^- current which ceased upon
superfusion with drug-free saline (see Fig. 20). Responses
to neomycin B were observed at concentrations between
100 μM and 10 mM. To ensure that the response evoked by
neomycin B was contingent upon injection of parathyroid
mRNA, the effect of neomycin B on currents in water-injected
oocytes was determined. In each of five oocytes examined,
neomycin B (10 mM) failed to cause any change in the
current. About 40% of oocytes are known to respond to
carbachol, an effect mediated by an endogenous muscarinic
receptor. In five oocytes examined, one showed inward

currents in response to carbachol, and this is shown in the
lower trace of Fig. 20. Thus, in cells expressing a
muscarinic receptor coupled to increases in $[Ca^{2+}]_i$ and
Cl^- current, neomycin B fails to evoke a response. This
shows that the response to neomycin B depends on expression
of a specific protein encoded by parathyroid cell mRNA. It
suggests quite strongly that in intact cells, neomycin B
acts directly on the Ca^{2+} receptor to alter parathyroid
cell function.

Drug Design From Lead Molecules

Certain organic molecules mimic or antagonize the
action of extracellular Ca^{2+} by acting at the Ca^{2+}
receptor as shown herein. The molecules tested, however,
are not necessarily suitable as drug candidates, but they
serve to demonstrate that the hypothesis underlying Ca^{2+}
receptor-based therapies is correct. These molecules can be
used to determine the structural features that enable them
to act on the Ca^{2+} receptor, and thus to select molecules
useful in this invention.

An example of one such analytical procedure
follows: This example is detailed in the examples below,
but is used here to demonstrate the rationale that can be
used to design useful molecules of this invention from lead
molecules discussed herein. Those in the art will recognize
the analytic steps defined in the example and that analogous
analysis can be conducted on other lead molecules until the
most desired calcimimetic or calcilytic is defined.

Other examples are also provided below. Together
the data presented demonstrate that useful lead molecules
will have aromatic groups which are preferably substituted

- 55 -

at one or more positions, and may have branched or linear
substituted or unsubstituted alkyl groups as desired. In
addition, it is important to choose molecules of correct
stereospecificity to ensure higher affinity for the desired
Ca^{2+} receptor. These data thus point those in the art to
appropriate lead molecules which can be derivatised to find
optimum desired molecules of this invention, much as
described below.

Although structurally diverse, molecules that are
tested may have common features that can be studied. In
this example, the correlation between net positive charge
and potency in mobilizing intracellular Ca^{2+} was tested.
Protamine (+21; EC_{50} = 40 nM) was more effective than
neomycin B (+6; EC_{50} = 20 µM in human parathyroid cells
and 40 µM in bovine parathyroid cells) which was more
effective than spermine (+4; EC_{50} = 150 µM) in causing
the mobilization of $[Ca^{2+}]_i$ in parathyroid cells. These
results raise the question of whether positive charge alone
determines potency, or if there are other structural
features that contribute to activity on the Ca^{2+}
receptor. This is important to determine at the outset
because it profoundly impacts on the view that the Ca^{2+}
receptor can be targeted with effective and specific
therapeutic molecules. Thus, a variety of other organic
polycations related to neomycin B and spermine can be
studied to determine the relationship between the net
positive charge of a molecule and its potency to mobilize
intracellular Ca^{2+}.

The first series of molecules studied were the
aminoglycosides. The molecules were examined on bovine

parathyroid cells and their EC_{50}'s for the mobilization of
intracellular Ca^{2+} determined. For the aminoglycosides,
the rank order of potency for eliciting cytosolic Ca^{2+}
transients was neomycin B (EC_{50} = 20 or 40 µM) >
gentamicin (150 µM) > bekanamycin (200 µM) >
streptomycin (600 µM). Kanamycin and lincomycin were
without effect when tested at a concentration of 500 µM.
The net positive charge on these aminoglycosides at pH 7.3
is neomycin B (+6) > gentamicin (+5) = bekanamycin (+5) >
kanamycin (average +4.5) > streptomycin (+3) > lincomycin
(+1). Within the aminoglycoside series, then, there is some
correlation between net positive charge but it is not
absolute, and kanamycin, which would be predicted to be more
potent than streptomycin, is without activity.

Testing of various polyamines revealed additional
and more marked discrepancies between net positive charge
and potency. Three structural classes of polyamines were
examined: (1) straight chain, (2) branched chain, and (3)
cyclic. The structures of the polyamines tested are
provided in Fig. 1. Amongst the straight chain polyamines,
spermine (+4; EC_{50} = 150 µM) was more potent than
pentaethylenehexamine (+6; EC_{50} = 500 µM) and
tetraethylenepentamine (+5; EC_{50} = 2.5 mM) even though the
latter molecules have a greater net positive charge.

We synthesized some branched chain polyamines that
have different numbers of secondary and primary amino groups
and thus vary in net positive charge. Two of these
molecules, NPS 381 and NPS 382, were examined for effects on
$[Ca^{2+}]_i$ in bovine parathyroid cells. NPS 382 (+8;
EC_{50} = 50 µM) was about twice as potent as NPS 381 (+10;

EC_{50} = 100 μM) even though it contains two fewer
positive charges.

A similar discrepancy between positive charge and
potency was noted in experiments with cyclic polyamines.
For example, hexacyclen (+6; EC_{50} = 20 μM) was more
potent than NPS 383 (+8; EC_{50} = 150 μM). The results
obtained with these polyamines show that positive charge is
not the sole factor contributing to potency.

Additional studies provided insights into the
structural features of molecules that impart activity on the
parathyroid cell Ca^{2+} receptor. One of the structurally
important features is the intramolecular distance between
the nitrogens (which carry the positive charge). Thus,
spermine is 50-fold more potent than triethylenetetramine
(EC_{50} = 8 mM) in evoking increases in $[Ca^{2+}]_i$ in
bovine parathyroid cells yet both molecules carry a net
positive charge of +4. The only difference in structure
between these two polyamines is the number of methylenes
separating the nitrogens: in spermine it is 3-4-3 whereas in
triethylenetetramine it is 2-2-2. This seemingly minor
change in the spacing between nitrogens has profound
implications for potency and suggests that the
conformational relationships of nitrogens within the
molecule are critical. Supporting this are results obtained
with hexacyclen and pentaethylenehexamine. The former
molecule is simply the cyclic analog of the latter and
contains the same number of methylenes between all
nitrogens, yet the presence of the ring structure increases
potency 25-fold. These results indicate that positive
charge per se is not the critical factor determining the

- 58 -

activity of an organic molecule on the Ca^{2+} receptor.

Another series of experiments reveals the importance of aromatic groups in determining activity on the Ca^{2+} receptor. The results were obtained with two arylalkylamines isolated from the venom of the spider Argiope lobata. These molecules, argiotoxin 636 and argiotoxin 659, have identical polycationic portions linked to different aromatic groups (Fig. 24). Argiotoxin 659 evoked transient increases in [Ca^{2+}]$_i$ in bovine parathyroid cells when tested at concentrations of 100 to 300 µM. In contrast, argiotoxin 636 was without effect when tested at similar concentrations (Fig. 24). The only difference in structure between these two arylalkylamines is in the aromatic portion of the molecules: argiotoxin 659 contains a 4-hydroxyindole moiety whereas argiotoxin 636 contains a 2,4-dihydroxyphenyl group. The net positive charge on these two arylalkylamines is the same (+4), so their different potencies must result from the different aromatic groups. This shows that net positive charge alone does not determine potency. The real importance of these findings, however, is the discovery that aromatic groups contribute significantly to the ability of molecules to activate the Ca^{2+} receptor.

Agatoxin 489 (NPS 017) and Agatoxin 505 (NPS 015) both cause the mobilization of intracellular Ca^{2+} in parathyroid cells with EC$_{50}$'s of 6 and 22 µM, respectively. The only difference in the structure of these molecules is a hydroxyl group on the indole moiety (Fig. 1). This shows that substitutions on the aromatic region of the molecule can influence potency. This

- 59 -

indicates that further lead molecules to be studied will include those molecules having substituted aromatic moieties. The structural features to be varied systematically from lead molecules described herein include (1) net positive charge, (2) number of methylenes separating nitrogens, and (3) cyclic versions of, e.g., polyamines, with and without changes in methylene spacing and net positive charge. In addition systematic variations in the structure and location of aromatic groups can be examined, e.g., in a variety of arylalkylamines isolated from the venoms of wasps and spiders; and synthetic molecules can be prepared by the coupling of commercially available aromatic moieties to the argiotoxin polyamine moiety. The argiotoxin polyamine moiety can be readily coupled to any aromatic moiety containing a carboxylic acid. Thus, it is simple to systematically screen the hydroxy and methoxy derivatives of phenylacetic acid and benzoic acid as well as the hydroxyindoleacetic acid series. Analogs containing heteroaromatic functionalities can also be prepared and assessed for activity.

Comparisons of potency and efficacy among such molecules will reveal the optimal structure and location of the aromatic group at a constant positive charge.

One of the structural variations on the polyamine motif that seems to increase potency is the presence of the cyclic version of the straight chain- parent molecule. Budmunchiamine A, isolated from the plant Albizia amara, is a cyclic derivative of spermine (Fig. 1). The addition of budmunchiamine A to bovine parathyroid cells caused a rapid and transient increase in $[Ca^{2+}]_i$ that persisted in the

- 60 -

absence of extracellular Ca^{2+} and was blunted by
pretreatment with PMA. It therefore causes the mobilization
of intracellular Ca^{2+} in parathyroid cells, probably by
acting on the Ca^{2+} receptor. It is about equipotent with
spermine (EC_{50} about 200 µM) yet carries one less
positive charge (+3) than does spermine.

The results obtained with budmunchiamine A
demonstrate the predictive power of the structure-activity
studies and the novel structural information to be gained by
testing natural products. Thus, screening of natural
products, selected rationally on the basis of the structural
information is readily performed e.g., molecules can be
selected on the basis of well-established chemotaxonomic
principles using appropriate data bases, such as Napralert.
For example, macrocyclic polyamine alkaloids derived from
papilionoid legumes related to Albizia, such as
Pithecolobium and other plant-derived molecules can be
screened.

Fig. 36 provides a second example of a series of
molecules which were screened to determine useful molecules
of this invention. These molecules were generally derived
from fendiline and tested to determine their respective
EC_{50}'s. Moreover, testing of related molecules, such as
NPS 447 and NPS 448 reveals stereospecific effects of
molecule structure. The most active compounds tested to
date are the novel compounds designated NPS 467 and NPS 568
which have EC_{50} values of less than 5 µM. Those in the
art, by reviewing this series of molecules, can determine
other suitable derivatives which can be tested in the
invention.

- 61 -

These examples demonstrate the general design and
screening process useful in this invention, and indicate
that additional compound and natural product libraries can
be screened as desired by those in the art to determine
other useful lead molecules or novel molecules of this
invention.

As discussed above, examples of molecules useful
as calcimimetics include branched or cyclic polyamines,
positively charged polyamino acids, and arylalkylamines. In
addition, other positively charged organic molecules,
including naturally occurring molecules and their analogs,
are useful calcimimetics. These naturally occurring
molecules and their analogs preferably have positive
charge-to-mass ratios that correlate with those ratios for
the molecules exemplified herein. (Examples include
material isolated from marine species, arthropod venoms,
terrestrial plants and fermentation broths derived from
bacteria and fungi.) It is contemplated that one group of
preferred naturally occurring molecules and analogs useful
as calcimimetics will have a ratio of positive charge:
molecular weight (in daltons) from about 1:40 to 1:200,
preferably from about 1:40 to 1:100. More specific examples
of such molecules are provided below.

Polyamines

The polyamines useful as calcimimetics in this
invention may be either branched or cyclic. Branched or
cyclic polyamines potentially have higher calcimimetic
activity than their straight-chain analogs. That is,
branched or cyclic polyamines tend to have a lower EC_{50}
than their corresponding linear polyamines with the same
effective charge at physiological pH (see Table 1).

Table 1

Molecule	Net (+) Charge	$EC_{50}(\mu M)$
Neomycin	+6	20 or 40
Hexacyclen	+6	20
NPS 382	+8	50
NPS 381	+10	100
NPS 383	+8	150
Gentamicin	+5	150
Spermine	+4	150
Bekanamycin	+5	200
Argiotoxin-659	+4	300
Pentaethylenehexamine (PEHA)	+6	500
Streptomycin	+3	600
Spermidine	+3	2000
Tetraethylenepentamine (TEPA)	+5	2500
1,12-diaminododecane (DADD)	+2	3000
Triethylenetramine (TETA)	+4	8000

"Branched polyamines" as used herein refers to a chain molecule consisting of short alkyl bridges or alkyl groups joined together by amino linkages, and also containing points at which the chain branches. These "branch points" can be located at either a carbon atom or a nitrogen atom, preferably at a nitrogen atom. A nitrogen atom branch point is typically a tertiary amine but it may also be quaternary. A branched polyamine may have 1 to 20 branch points, preferably 1 to 10 branch points.

Generally, the alkyl bridges and alkyl branches in a branched polyamine are from 1 to 50 carbon atoms in length,

- 63 -

preferably from 2 to 6 carbon atoms. The alkyl branches may also be interrupted by one or more heteroatoms (nitrogen, oxygen or sulfur) or substituted with functional groups such as: halo, including fluoro, chloro, bromo, or iodo; hydroxy; nitro; acyloxy (R'COO-), acylamido (R'CONH-), or alkoxy (-OR'), where R' may contain from 1 to 4 carbon atoms. The alkyl branches may also be substituted with groups that are positively charged at physiological pH, such as amino or guanido. These functional substituents may add or change physical properties such as solubility to increase activity, delivery or bioavailability of the molecules.

The branched polyamines may have three or more chain and branch termination points. These termination points may be methyl groups or amino groups, preferably amino groups.

One preferred group of molecules is the group of branched polyamines having the formula:
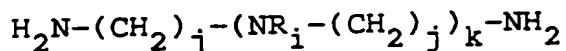
$$H_2N-(CH_2)_j-(NR_i-(CH_2)_j)_k-NH_2$$

where k is an integer from 1 to 10, each j is the same or different and is an integer from 2 to 20, and each R_i is the same or different and is selected from the group consisting of hydrogen and $-(CH_2)_j-NH_2$, where j is as defined above, and at least one R_i is not hydrogen.

Particularly preferred branched polyamines of this invention are the molecules $N^1,N^1,N^5,N^{10},N^{14},N^{14}$-hexakis-(3- aminopropyl) spermine and $N^1,N^1,N^5,N^{14},N^{14}$-tetrakis-(3-aminopropyl)spermine referred to as NPS 381 and NPS 382, respectively, in Figure 1.

"Cyclic polyamines" as used herein refer to heterocycles containing two or more heteroatoms (nitrogen,

- 64 -

oxygen or sulfur), at least two of which are nitrogen
atoms. The heterocycles are generally from about 6 to about
20 atoms in circumference, preferably from about 10 to about
18 atoms in circumference. The nitrogen heteroatoms are
separated by 2 to 10 carbon atoms. The heterocycles may
also be substituted at the nitrogen sites with aminoalkyl or
aminoaryl groups (NH_2R-), wherein R is aminoaryl or a
lower alkyl of 2 to 6 carbon atoms.

Particularly preferred cyclic polyamines of this
invention are shown in Figure 1 as hexacyclen
(1,4,7,10,13,16-hexaaza-cyclooctadecane) and NPS 383.

Polyamino Acids

The polyamino acids useful in this invention may
contain two or more positively charged amino acid residues
at physiological pH. These positively charged amino acids
include histidine, lysine and arginine. These polypeptides
will vary in length from 2 to 800 amino acids in length,
more preferably from 20 to 300 amino acids in length. These
polypeptides may consist of a single repeating amino acid
residue, or may have the variety of a naturally occurring
protein or enzyme.

The amino acid residues comprising the polyamino
acids may be any of the twenty naturally occurring amino
acids, or other alternative residues. Alternative residues
include, for example, the   ositions of this invention may
also contain components derivatized with a molecule or ion
which acts as a label. A wide variety of labeling moieties
can be used, including radioisotopes, chromophores, and
fluorescent labels. Radioisotope labeling in particular can
be readily detected in vivo. Radioisotopes may be coupled

by coordination as cations in the porphyrin system. Useful
cations include technetium, and indium. In the
compositions, the positively charged molecule can be linked
to or associated with a label.

Methods of Synthesis

Strategies for the syntheses and the modification of
polyamines involve the use of a variety of amine protecting
groups (phthalimido, BOC, CBZ, benzyl, and nitrile) which
can be removed selectively to construct functionalized
molecules. The synthetic methods involved are modelled
after those used to construct argiopines 636 and 659 and
other arylalkylamines derived from spider venoms.

Chain extensions of 2-4 methylenes were typically
accomplished by alkylation with the corresponding
N-(bromoalkyl)phthalimide. A 1:1.2 mixture of amine to the
bromoalkylphthalimide was refluxed in acetonitrile in the
presence of 50% KF on Celite. Chain extensions were also
accomplished by alkylation of a given amine with
acrylonitrile or ethylacrylate. Reaction progress was
monitored by TLC and intermediates purified on silica gel
using combinations of dichloromethane, methanol, and
isopropylamine. Final products were purified by cation
exchange (HEMA-SB) and RP-HPLC (Vydac C-18). Purity and
structure verification are accomplished by ^{1}H- and
^{13}C-NMR and high-resolution mass spectrometry (EI, CI
and/or FAB).

BOC protecting groups were added by the treatment of
an amine (1° or 2°) with di-tert-butyl dicarbonate in
dichloromethane in the presence of a catalytic amount of
dimethylaminopyridine. Benzyl protecting groups were

applied in one of two ways: (1) condensation of a 1^O amine
with benzaldehyde followed by sodium borohydride reduction
or (2) alkylation of a 2^O amine with benzylbromide in the
presence of KF. Amide linkages and cyclizations were
typically performed by the reaction of an amine (1° or 2°)
with the N-hydroxysuccinimide ester of a given acid. This
was accomplished directly (in the case of cyclizations) by
treatment of the "amino acid" with dicyclohexylcarbodiimide
under dilute conditions.

Deprotections of the phthalimido functionality were
accomplished by reduction with hydrazine in refluxing
methanol. Deprotections of the BOC functionality were
accomplished in anhydrous TFA. Deprotection of benzyl,
nitrile, and CBZ protecting functionalities was accomplished
by reduction in glacial acetic acid under 55 psi hydrogen in
the presence of a catalytic amount of palladium hydroxide on
carbon. Nitrile functionalities (in the presence of benzyl
and CBZ groups) were selectively reduced under hydrogen in
the presence of sponge Raney nickel.

Specifically, branched polyamines are typically
prepared from simple diaminoalkanes of the formula NH_2-
$(CH_2)_n$-NH_2, or simple polyamines such as spermidine or
spermine. One of the two primary (terminal) amines is
protected or "masked" with a protecting group such as BOC
(t-butyloxycarbonyl), phthalimido, benzyl, 2- ethylnitrile
(the Michael condensation production product of an amine and
acrylonitrile), or amide. A typical reaction is the
addition of a BOC protecting group by treatment with
di-t-butyl-dicarbonate (BOC anhydride):

$$H_2N-(CH_2)_n-NH_2 \xrightarrow{\text{BOC anhydride}} \begin{array}{c} H_2N-(CH_2)_n-NHBOC \\ + \\ BOCHN-(CH_2)_n-NHBOC \end{array}$$
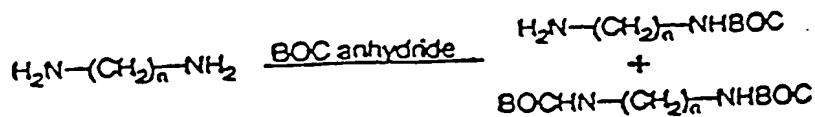
The monoprotected product is separated from the unprotected and diprotected products by simple chromatographic or distillation techniques.

The remaining free amine in the monoprotected product is then selectively alkylated (or acylated) with an alkylating (or acylating) agent. To ensure mono-alkylation, the free amine is partially protected by condensation with benzaldehyde followed by sodium borohydride reduction to form the N-benzyl derivative:

$$H_2N-(CH_2)_n-NHBOC \xrightarrow[\text{2) NaBH}_4]{\text{1) PhCHO}} \underset{\text{Ph}}{HN}-(CH_2)_n-NHBOC$$
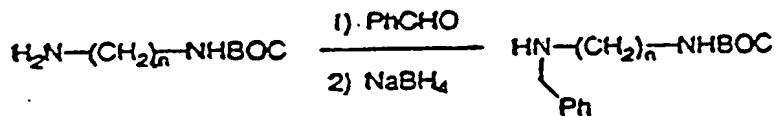
The N-benzyl derivative is then reacted with the alkylating agent. A typical alkylating agent is in an N-(bromoalkyl)phthalimide, which reacts as follows:
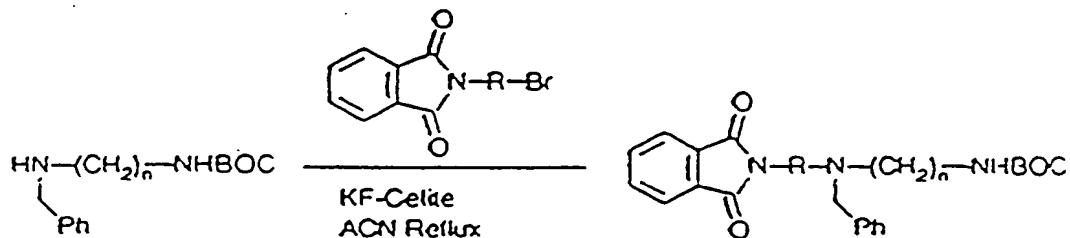
For example, N-(bromobutyl)phthalimide is used to extend or branch the chain with four methylene units. Alternatively,

reaction with acrylonitrile followed by reduction of the
cyano group will extend the chain by three methylenes and an
amino group.

The protecting groups of the resulting chain-
extended molecule can then be selectively cleaved to yield a
new free amine. For example, trifluoroacetic acid is used
to remove a BOC group; catalytic hydrogenation is used to
reduce a nitrile functionality and remove a benzyl group;
and hydrazine is used to remove phthalimido groups as
follows:
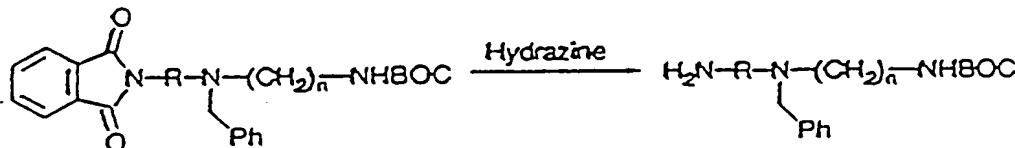
The new free amine may be alkylated (or acylated)
further as above to increase the length of the polyamine.
This process is repeated until the desired chain length and
number of branches is obtained. In the final step,
deprotection of the product results in the desired
polyamine. However, further modifications may be effected
at the protected end prior to deprotection in the following
manner:

For example, prior to BOC-deprotection, the polyamine
is acylated with the N-hydroxysuccinimide ester of
3,4-dimethoxyphenylacetic acid to yield a diprotected
polyamine:
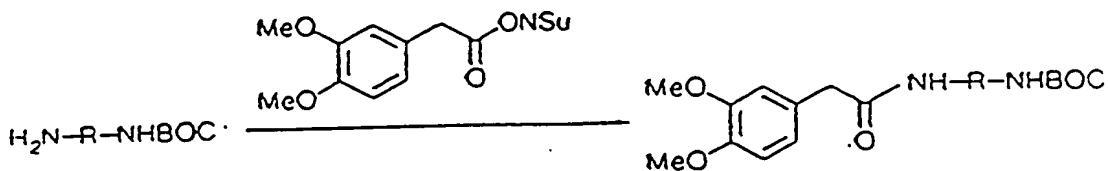
- 69 -

This ultimately will yield an arylalkyl polyamine.
The BOC group can then be selectively removed with
trifluoroacetic acid to expose the other amino terminus
which can be extended as above.

Certain branched polyamines may be formed by
simultaneously alkylating or acylating the free primary and
secondary amines in a polyamine formed as above. For
example, treatment of spermine with excess acrylonitrile
followed by catalytic reduction yields the following:
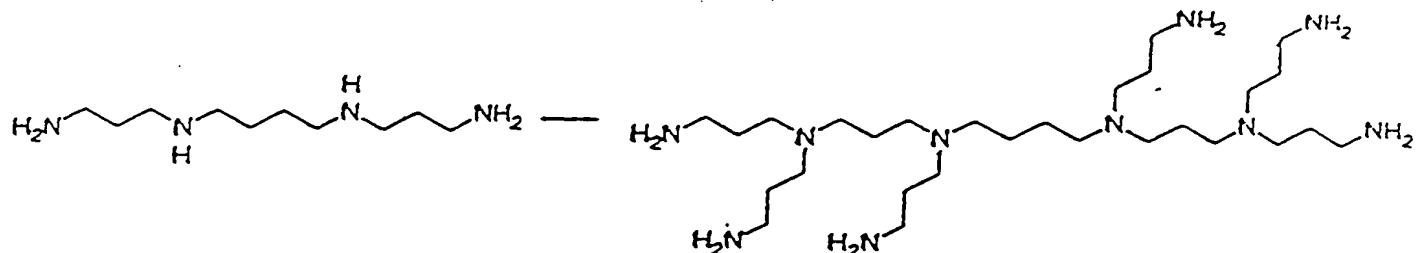
Cyclic polyamines may be prepared as above beginning
with starting materials such as hexacylen (Aldrich Chem.).

The polyamino acids within the scope of the present
invention can be made by recombinant techniques known in the
art, or may be synthesized using standard solid-phase
techniques known in the art. Solid-phase synthesis is
commenced from the carboxy-terminal end of the peptide using
an μ-amino protected amino acid. BOC protective groups
can be used for all amino groups even through other
protective groups are suitable. For example, BOC-lys-OH can
be esterified to chloromethylated polystyrene resin
supports. The polystyrene resin support is preferably a
copolymer of styrene with about 0.5 to 2% divinylbenzene as

- 70 -

a cross-linking agent which causes the polystyrene polymer
to be completely insoluble in certain organic solvents. See
Stewart et al., Solid-Phase Peptide Synthesis (1969), W.H.
Freeman Co., San Francisco; and Merrifield, J. Am. Chem.
Soc. (1963) 85:2149-2154. These and other methods of
peptide synthesis are also exemplified by U.S. Patent Nos.
3,862,925; 3,842,067; 3,972,859; and 4,105,602.

   The polypeptide synthesis may use manual techniques
or automatically employing, for example, an Applied
Biosystems 403A Peptide Synthesizer (Foster City,
California) or a Biosearch SAM II automatic peptide
synthesizer (Biosearch, Inc., San Rafael, California),
following the instructions provided in the instruction
manual supplied by the manufacturer.

   The arylalkylamines of the invention are natural
products isolated by known techniques, or synthesized as
described in Jasys et al., Tetrahedron Lett. (1988)
29:6223-6226, and Nason et al., Tetrahedron Lett. (1989)
30:2337-2340.

   One general protocol for preparation of fendiline (or
fendiline analogs shown in Fig. 36) is as follows. In a 10
ml round bottom flask equipped with a magnetic stir bar and
rubber septum, 1.0 mmole 3,3'- bisphenylpropylamine (or
primary alkyl amine) in 2 ml ethanol was treated with 1.1
mmole phenol and 1.0 mmole acetophenone (or substituted
acetophenone). To this was added 2.0 mmoles $MgSO_4$ and 1.0
mmole $NaCNBH_3$. This was stirred under a nitrogen
atmosphere at room temperature (about 20°C) for 24 hrs. The
reaction was poured into 50 ml ether and washed 3 times with
1 N NaOH and once with brine. The ether layer was dried

with anhydrous K_2CO_3 and reduced _in vacuo_. The product
was then purified by column chromatography or HPLC
incorporating osilica stationary phase with combinations of
CH_2Cl_2- Methanol- isopropylamine (typically 3% Methanol
and 0.1% isopropylamine in methylene chloride).

A preferred procedure for preparing fendiline or
fendiline analogs (such as those depicted in Figure 36) uses
titanium(IV) isopropoxide and was modified from methods
described in _J. Org. Chem._ __55__:2552 (1990). For the
synthesis of NPS 544, titanium tetrachloride (method
described in _Tetrahedron Letters_ __31__:5547 (1990)) was used in
place of titanium(IV) isopropoxide. The reaction scheme is
depicted in Figure 43a. In Figure 43a, R,R' and R" depict
hydrocarbyl groups. According to one embodiment, in a 4 ml
vial, 1 mmole of amine (__1__) (typically a primary amine) and
1 mmole ketone or aldehyde (__2__) (generally acetophenone) are
mixed, then treated with 1.25 mmoles titanium(IV)
isopropoxide (__3__) and allowed to stand with occasional
stirring at room temperature for about 30 minutes.
Alternatively, a secondary amine may be used in place of
(__1__). Note: some reactions will give heavy precipitates or
solids which are warmed/heated (to their melting point) to
allow for stirring/mixing several times over the course of
the reaction. The reaction mixture is treated with 1 ml
ethanol containing 1 mmole sodium cyanoborohydride (__4__) and
the resulting mixture is then allowed to stand at room
temperature with occasional stirring for about 16 hours.
After this time the reaction is quenched by the addition of
about 500 μl water. The reaction mixture is then diluted
to about 4 ml total volume with ethyl ether and then

- 72 -

centrifuged. The upper organic phase is removed and reduced
on a rotavapor. The resulting product, (6), is partially
purified by chromatography through a short column of silica
(or alternatively by using preparative TLC on silica) using
combination of dichloromethane:methanol:isopropylamine
(typically 95:5:0.1), prior to purification by HPLC (normal
phase using silica with dichloromethane:methanol:
isopropylamine or reversed phase, C-18 with 0.1% TFA with
acetonitrile or methanol).

     If appropriate or desired, chiral resolution may be
accomplished using methods such as those described in
Example 21.

Formulation and Administration

     As demonstrated herein, the molecules of the
invention may be used to: (a) mimic or antagonize one or
more of the effect of an extracellular ion, including
extracellular Ca^{2+}; (b) affect the extracellular free
Ca^{2+} level in an individual; and (c) treat diseases such
as hyperparathyroidism, osteoporosis and hypertension. In
general, diseases or conditions involving inorganic-ion
receptors now can be studied, diagnosed and/or beneficially
treated. While the molecules have generally been shown to
have an effect on parathyroid cells, they may also modulate
the Ca^{2+} receptors on other cells, including bone
osteoclasts, juxtaglomerular kidney cells, proximal tubule
kidney cells, distal tubule kidney cell, cell of the thick
ascending limb of Henle's loop and/or collecting duct,
keratinocyte in the epidermis, parafollicular cell in the
thyroid (C-cells), intestinal cell, trophoblast in the
placenta, platelet, vascular smooth muscle cell, cardiac

- 73 -

atrial cell, gastrin and glucagon secreting cells, kidney mesangial cell and mammary cell.

While these molecules will typically be used in therapy for human patients, they may be used to treat similar or identical diseases in other warm-blooded animal species such as other primates, farm animals such as swine, cattle, and poultry; and sports animals and pets such as horses, dogs and cats.

In therapeutic and/or diagnostic applications, the molecules of the invention can be formulated for a variety of modes of administration, including systemic and topical or localized administration. Techniques and formulations generally may be found in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, PA.

For systemic administration, oral administration is preferred. Alternatively, injection may be used, e.g., intramuscular, intravenous, intraperitoneal, and subcutaneous. For injection, the molecules of the invention are formulated in liquid solutions, preferably in physiologically compatible buffers such as Hank's solution or Ringer's solution. In addition, the molecules may be formulated in solid form and redissolved or suspended immediately prior to use. Lyophilized forms are also included.

Systemic administration can also be by transmucosal or transdermal means, or the molecules can be administrated orally. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal

- 74 -

administration bile salts and fusidic acid derivatives. In
addition, detergents may be used to facilitate permeation.
Transmucosal administration may be through nasal sprays, for
example, or using suppositories. For oral administration,
the molecules are formulated into conventional oral
administration forms such as capsules, tablets, and tonics.

For topical administration, the molecules of the
invention are formulated into ointments, salves, gels, or
creams, as is generally known in the art.

As shown in the examples below, the amounts of
various compounds of this invention which must be
administered can be determined by standard procedures.
Generally it is an amount between about 1 and 50 mg/kg
animal to be treated.

Recombinant Receptors

Natural product screening has traditionally provided
the lead structures for the development of diverse
therapeutic molecules. However, high-throughput screening
of natural product libraries or other molecule libraries for
activity on the Ca^{2+} receptor has not previously been
possible. To achieve this capability, it is best to clone
the Ca^{2+} receptor cDNA and then create transfected cell
lines suitable for high-throughput screening. The structure
of the receptor can additionally be used to gain insight
into the molecular geometry of the ligand binding site(s),
and such information used to augment a rational drug design
program as discussed above. Limited structure-activity
studies and testing of selected natural product molecules
will provide the initial structural data base necessary to
guide rational natural product screening and drug design.

The discovery of a superfamily of genes permits the
same advantages in connection with all inorganic-ion
receptors. Although the following discussion will refer
often to methodology for cloning a calcium receptor, it will
be understood by those of ordinary skill in the art that the
methodology generally is applicable to the cloning of all
inorganic-ion receptors.

These recombinant receptors allow for the first time
screening mimics and lytics, including calcimimetics and
calcilytics. For example, by binding assays with the
receptors. This is particularly useful in screening with
the human receptors in order to identify therapeutically
useful compounds.

The bovine and human parathyroid cell Ca^{2+} receptor
cDNA can be cloned by functional expression in <u>Xenopus</u>
oocytes. It is possible to monitor an increase in
intracellular Ca^{2+} in <u>Xenopus</u> oocytes indirectly by
measuring current through the endogenous Ca^{2+}-activated
Cl^- channel. The amplification of the response afforded
by this signal transduction pathway enables the detection of
receptor proteins encoded by mRNA at very low levels. This
allows the detection of receptor- specific cDNA clones
without the need for high affinity ligands, specific
antisera, or protein or nucleic acid sequence information.
An example of such a procedure follows.

Adult female <u>Xenopus</u> <u>laevis</u> were obtained from
<u>Xenopus</u> I (Ann Arbor, MI) and maintained according to
standard procedures. Lobes of ovary were excised from
hypothermically-anesthetized toads. Clusters of oocytes
were transferred into modified Barth's saline (MBS).

- 76 -

Individual oocytes were obtained by incubation in MBS
containing 2 mg/ml collagenase (Sigma, Type 1A) for 2h at
21°C and stage V-VI oocytes were selected for injection.

Glass capillary tubes (1 mm diameter) were pulled to
a fine tip and manually broken to achieve a tip diameter of
about 15 µM. A droplet of mRNA (1 ng/nl in
diethylpyrocarbonate (DEPC)-treated water) was placed onto
parafilm and drawn into the capillary tube by suction. The
capillary tube was then connected to a picospritzer (WPI
Instruments) and the volume of the air-pulsed droplets
adjusted to deliver 50 ng of mRNA (typically 50 nl). A 35
mm culture dish with a patch of nylon stocking fixed to the
bottom was used to secure the oocytes during injection of
mRNA into the vegetal pole. The injected oocytes were
placed into a 35 mm culture dish containing MBS, 100 µg/ml
penicillin and 100 µg/ml streptomycin and incubated at
18°C for 3 days.

Following incubation, an oocyte was placed into a 100
µl plastic chamber and superfused with MBS at a flow rate
of 0.5 ml/min using a peristaltic pump. Test molecules or
inorganic polycations were added by rapidly moving the
tubing into different buffers. Recording and
current-passing electrodes were constructed from thin wall
capillary tubing pulled to a resistance of 1-3 Mohms and
filled with 3 M KCl. Oocytes were impaled (in the animal
pole) with both electrodes under microscopic observation and
connected to an Axon Instruments Axoclamp 2A voltage-clamp
amplifier which was used to set the holding potential (-70
to -80 mV) and to measure the currents that were passed to
maintain the holding potential. Currents were recorded

- 77 -

directly onto a strip chart recorder.

For mRNA preparation, tissue was obtained from calves or patients with secondary HPT undergoing surgical removal of the parathyroid glands. Purified cells need not be prepared; whole pieces of gland were used to prepare mRNA that directs the expression of the Ca^{2+} receptor in Xenopus oocytes. Total cellular RNA was prepared by acid guanidinium thiocyanate/phenol extraction of homogenized glands. Oligo-dT cellulose chromatography was used to select poly(A)$^+$ mRNA by standard procedures. For size fractionation of mRNA, centrifugation through glycerol gradients was used. The mRNA was denatured with 20 mM methylmercuric hydroxide and loaded (50-100 μg at a concentration of 1 mg/ml) onto a linear 15-30% glycerol gradient prepared in Beckman TLS55 tubes. Following centrifugation at 34,000 rpm for 16 hrs, 0.3 ml gradient fractions were collected and diluted in an equal volume of water containing 5mM beta-mercaptoethanol. mRNA was then recovered by two cycles of ethanol precipitation. If desired, the mRNA (50-100 μg of poly(A)$^+$) may be separated on a 1.2% agarose/6.0 M urea preparative gel, along with a range of RNA size markers. Following visualization of the mRNA by ethidium bromide staining, gel slices containing RNA in approximately 1 kb to 2kb size steps are excised. mRNA is recovered from the agarose gel slices using RNAid binding matrix (according to the supplier's standard protocol; Stratagene, Inc.) and recovered mRNA fractions eluted into DEPC-treated water.

Amounts of recovered mRNA were quantified by UV absorbance measurement. The size range of mRNA contained

- 78 -

within each fraction of the glycerol gradient was determined
by formaldehyde/agarose gel electrophoresis using a small
quantity (0.5 µg) of each sample. The integrity of the
mRNA was assessed by in vitro translation of each sample.
Reticulocyte lysates (commercially available kits; BRL) were
used to translate 0.05-0.5 µg of each mRNA fraction. The
resulting ^{35}S-labelled proteins were analyzed by
SDS-PAGE. The intact mRNA was capable of directing the
synthesis of proteins of a complete size range,
corresponding roughly to the sizes of the individual mRNA
fractions.

A cDNA library was constructed in the vector λ
ZAPII, following modifications of the technique of Gubler
and Hoffman. RNA from the fraction(s) giving the best
response in the oocyte assay was used as starting material.
First-strand cDNA syntheses was primed with an oligo-dT/NotI
primer-linker. Second-strand synthesis was by the RNase
H/DNA Polymerase I self-priming method. Double-stranded
cDNA was blunted with T4 DNA polymerase and EcoRI adaptors
blunt-end ligated to the cDNA with T4 ligase. Following
NotI digestion to cleave the linker, full-length cDNA was
size-selected by exclusion chromatography on Sephacryl
500 HA. First-strand cDNA was radiolabeled with
α-^{32}P-dATP, and all synthesis and recovery steps
monitored by following the incorporation of radioactivity.
Full-length cDNA recovered from the sizing column was
ligated to EcoRI/NotI digested λ ZAPII arms. The ligation
mix was test packaged with commercially available
high-efficiency packaging extract (Stratagene, Inc.) and
plated on the appropriate host strain (XL1-blue). The

percentage of recombinant phage was determined by the ratio
of white-to-blue plaques when the library is plated on IPTG
and X-gal.

The average insert size was determined from ten
randomly selected clones. Phage DNA "mini-preps" were
digested with EcoRI and NotI to release the insert, and the
size determined by agarose gel electrophoresis. The library
consisted of >90% recombinant phage, and the insert size
ranged from 1.5 to 4.2 kb. The recombinant ligation was
packaged in large scale to generate 800,000 primary clones.
The packaging mix was titered and plated at 50,000 plaques
per 15 cm plate. Each pool of 50,000 clones was eluted in
SM buffer and stored individually.

Plate lysate stocks of each of the clone pools were
used for small-scale phage DNA preparation. Phage particles
are concentrated by polyethylene glycol precipitation, and
phage DNA purified by proteinase K digestion followed by
phenol:chloroform extraction. Twenty micrograms of DNA are
digested with NotI, and used as template for in vitro
transcription of sense-strand RNA. In vitro transcription
is according to standard protocols, utilizing T7 RNA
polymerase and 5' cap analog m^7GpppG in a 50 µl total
reaction volume. Following Dnase I/Proteinase K digestion
and phenol/chloroform extraction, the RNA is concentrated by
ethanol precipitation and used for oocyte injection.

Oocytes were injected with synthetic mRNA (cRNA) from
each of the 16 library subpools constituting 50,000
independent clones each. After incubation for 3 to 4 days,
oocytes were assayed for the ability of 10 mM neomycin to
elicit a Ca^{2+}-dependent Cl-current. A pool designated

"pool 6" gave a positive signal and thus contains a cDNA
clone encoding a functional calcium receptor. In order to
decrease the complexity of pool 6 and thus proceed towards
the purification of the calcium receptor clone contained
within this pool, pool 6 phage were replated at μ20,000
plaques per plate and 12 plates harvested. DNA was prepared
from each of these subpools and cRNA synthesized. Again,
oocytes were injected with cRNA and assayed 3-4 days later
for the ability of 10 mM neomycin to elicit a Ca^{2+-}
dependent Cl-current. A subpool pool 6-3 was positive and
this pool was subjected to a further round of plating,
reducing the complexity of pools to around 5,000 clones per
pool. Pools were again assayed by preparation of cRNA and
injection in oocytes. A subpool pool 6-3.4 was positive.
In order to expedite further purification of the positive
clone in pool 6-3.4, phage DNA from this pool was rescued as
plasmid DNA by superinfection with the helper phage,
ExAssist (Stratagene). Transfection of rescued plasmids
into bacterial strain DH5alphaF' resulted in transformed
bacterial colonies on ampicillin plates. These were
harvested in pool of 900 clones each. Plasmid DNA was then
prepared from each subpool and cRNA synthesized and assayed
in the usual manner. Subpool 6-3.4.4 was positive.
Bacteria containing the plasmid subpool 6-3.4.4 were
subsequently plated in subpools of μ50 clones each.
Continuation of this process is expected to result in a
single clone encoding a functional calcium receptor.

    Alternative assays are available for detecting
expression of a calcium receptor in oocytes. For example,
oocytes can be loaded with $^{45}Ca^{++}$ and then treated with

- 81 -

a calcimimetic. Mobilization of $^{45}Ca^{++}$ from
intracellular stores results in a net increase in $^{45}Ca^{++}$
efflux which is readily determined. Fluorescent Ca^{++}
indicators may also be injected into oocytes. In this case,
oocytes expressing a calcium receptor will exhibit increased
fluorescence upon activation with a calcimimetic. These
assays may be employed for cloning calcium receptors in
place of the electrophysiological assay for calcium induced
Cl^- current described in the above example. In addition,
the calcimimetic ligand used in the cloning procedure need
not be neomycin as indicated above but could instead be, for
example, Gd^{+++}, Ca^{++}, Mg^{++} or other calcimimetic
compound.

Initial experiments used Xenopus oocytes injected
with water or poly(A)$^+$-enriched mRNA (50 ng) from bovine
parathyroid cells. After three days, the oocytes were
examined for their ability to increase intracellular Ca^{2+}
in response to increases in the concentration of
extracellular di- and trivalent cations. The oocytes were
impaled with recording and current-passing electrodes and
$[Ca^{2+}]_i$ was assessed indirectly by measuring currents
through the endogenous Ca^{2+}-activated Cl^- channel. In
oocytes injected with poly(A)$^+$-enriched mRNA from bovine
(or human, Fig. 26) parathyroid cells, increasing the
concentration of extracellular Ca^{2+} from 0.7 to 3, 5 or 10
mM caused a rapid and transient increase in the Cl^-
current which then oscillated around a higher basal
current. Increasing the concentration of extracellular
Mg^{2+} from 1 to 10 mM likewise evoked oscillatory increases
in Cl^- current. The Cl^- current response to

extracellular Mg^{2+} persisted when the extracellular Ca^{2+} concentration was reduced to < 1 μM (Fig. 25).

The impermeant trivalent cation Gd^{3+} (600 μM) also caused oscillatory increases in the Cl^- current (Fig. 25). Such increases in the Cl^- current which oscillate and persist in the nominal absence of extracellular Ca^{2+} are noted when oocytes have been allowed to express other Ca^{2+}-mobilizing receptors and are stimulated with the appropriate ligand (e.g., substance K, Fig. 25). In these instances, the increase in Cl^- current reflects the mobilization of intracellular Ca^{2+}. These initial studies likewise show that extracellular polycations mobilize intracellular Ca^{2+} in parathyroid cell mRNA-injected oocytes.

Oocytes injected with water did not show any change in the Cl^- current when exposed to extracellular Ca^{2+} (10 mM) or Mg^{2+} (20 or 30 mM). In one series of experiments, oocytes were injected with the mRNA encoding the substance K receptor. In these oocytes, extracellular Mg^{2+} (20 mM) did not evoke any current but the cells responded vigorously to the addition of substance K (Fig. 25). These experiments indicate that there is no endogenous sensitivity of the oocyte to extracellular Ca^{2+} or Mg^{2+}.

Similar experiments were performed using oocytes injected with poly(A)$^+$-enriched mRNA prepared from human parathyroid glands (hyperplastic tissue from a case of secondary HPT). In these oocytes, increasing the concentration of extracellular Ca^{2+} caused a reversible increase in the Cl^- current which oscillated (Fig. 26). The addition of 300 μM La^{3+} likewise caused oscillatory

increases in the Cl^- current. Increasing the
concentration of extracellular Mg^{2+} from 1 to 10 mM evoked
increases in the Cl^- current that persisted in the absence
of extracellular Ca^{2+}. Additional experiments suggest
that the response to extracellular Ca^{2+} is concentration
dependent. Thus, in three mRNA-injected oocytes, Cl^-
current increased to a maximum of 111 $\pm$ 22 nA at 3 mM and
233 $\pm$ 101 nA at 10 mM extracellular Ca^{2+}.

The results obtained in Xenopus oocytes demonstrate
the presence of a mRNA(s) in parathyroid cells encoding a
protein(s) which can impart, in normally unresponsive cells,
sensitivity to extracellular Ca^{2+}. Moreover, the ability
of extracellular Mg^{2+} to evoke oscillatory increases in
Cl^- current in the absence of extracellular Ca^{2+}
demonstrates that the Cl^- current depends on the
mobilization of intracellular Ca^{2+} rather than influx of
extracellular Ca^{2+}. The results obtained with La^{3+}
likewise show that the expressed protein(s) is linked to the
mobilization of intracellular Ca^{2+}. Together, these data
show that the expressed protein(s) acts as a cell surface
receptor rather than a channel. These studies provide
compelling evidence for the existence of a Ca^{2+} receptor
protein on the surface of parathyroid cells and demonstrate
the feasibility of using the Xenopus oocyte system to
achieve the molecular cloning of the Ca^{2+} receptor cDNA.

In another series of experiments, parathyroid cell
mRNA was size fractionated by centrifugation through a
glycerol gradient. Ten fractions were collected. Each
group was injected into Xenopus oocytes and after a three
day incubation period the oocytes were assayed for

- 84 -

expression of the Ca^{2+} receptor. Those oocytes injected
with fractions 4-6 showed the largest and most consistent
increases in Cl^- current in response to extracellular
Ca^{2+} (Fig. 35). These results indicate that the Ca^{2+}
receptor is encoded by mRNA in a size range of 2.5-3.5 kb.
This indicates that a strategy using direct expression of
RNA synthesized from a transcription vector cDNA library is
feasible. Size-fractionation experiments of this sort were
conducted and in each of three different fractionation
experiments similar results were obtained.

The mRNA fractions obtained and characterized in the
preceding experiments can be assayed by injection into
oocytes. For each mRNA fraction, 10-20 oocytes are injected
with 50 ng of RNA at a concentration of 1 ng/nl in water.
Injected oocytes are maintained at 18° C for 48-72 h after
which they are assessed for expression of the Ca^{2+}
receptor using measurements of Cl^- current. For each
group of injected oocytes the number positive for expression
of the receptor, as well as the magnitude of the
Ca^{2+}-dependent Cl^- current measured, is determined. As
negative controls, oocytes are injected with rat liver
poly(A)$^+$-enriched mRNA, yeast RNA, or water.

It is expected that an mRNA in the range of 2.5 - 3.5
kb will encode the receptor. mRNA of a larger size may
necessitate a cloning approach based on hybrid depletion of
parathyroid mRNA prior to oocyte injection. This strategy
is not dependent upon the generation of full-length cDNA
clones for success. If receptor expression is not obtained
with a single size fraction of mRNA, oocytes are injected
with mixed size fractions to determine a combination that

- 85 -

does give rise to a functional receptor. If it does appear
that multiple subunits are necessary for the formation of a
functional receptor, the hybrid depletion expression cloning
strategy is used. In this approach, clones are selected on
the basis of their ability to deplete a specific mRNA
species from the total mRNA population. A clone encoding a
single subunit is identified by its ability to prevent the
formation of the active multi-subunit complex. By
exhaustive screening it is possible to identify clones
encoding all of the necessary subunits.

This approach permits the isolation of clones
encoding individual subunits required to form a functional
receptor complex. Synthetic RNA from pools of clones are
assayed for their ability to induce expression of the Ca^{2+}
receptor in Xenopus oocytes by the same techniques used to
analyze the original mRNA fractions. Originally, 10 pools
representing 100,000 primary clones each are examined.
Pools of clones showing a positive response are screened at
lower (typically 4-to-10 fold) complexity, and again
positive pools further subdivided and screened. This
process of library sub-fractionation is followed until
individual positive clones are identified. As a negative
control for the oocyte expression assay, anti-sense
transcripts are generated from those DNA templates that
induce a positive response. Anti-sense transcripts are
unable to give rise to an authentic receptor, and this will
control any non-specific positive signal arising from
injection of synthetic RNA. Another concern is the fact
that synthetic RNA can occasionally "poison" translation in
injected oocytes, by an undefined mechanism. To control for

this possibility, synthetic RNAs giving a negative response
are co-injected at various dilutions with parathyroid cell
mRNA, to determine if they are non-specifically interfering
with the expression of the Ca^{2+} receptor.

When an individual clone encoding the Ca^{2+} receptor
is identified, the cDNA insert will be excised from the
vector and used for large scale production of synthetic
RNA. Oocyte injection of this single RNA species allows
rigorous assessment of the characteristics of the expressed
receptor.

If the size of the mRNA encoding the Ca^{2+} receptor
is too large for cloning by direct transcription and
expression, or if multiple subunits are involved, a
hybrid-depletion technique of screening pools of clones is
used. cDNA insert DNA will be prepared from pools of clones
from the size-selected parathyroid cell cDNA library. This
DNA is hybridized to parathyroid cell mRNA under conditions
that permit the formation of DNA/RNA duplexes. The
unannealed, hybrid-depleted RNA is recovered and used for
oocyte injections. Other methods for hybrid-depletion or
hybrid-arrest may be used alternatively and are well known
by those skilled in the art. DNA from pools of clones
containing sequences representing Ca^{2+}-receptor mRNA is
depleted from this mRNA from the total parathyroid cell mRNA
population, and expression of the receptor is reduced or
absent upon oocyte injection. A process of
sub-fractionation is followed on pools of clones of
decreasing complexity, at each step assaying for cloned DNA
that depletes Ca^{2+} receptor-encoding mRNA from the total
parathyroid cell mRNA population. The use of an internal

- 87 -

control during the hybrid depletion assays ensures that the
hybrid-depleted RNA is intact and capable of being
translated in the oocyte.

Human parathyroid cells express a beta-adrenergic
receptor coupled to adenylate cyclase. This receptor can be
expressed in oocytes, where it is capable of agonist-induced
activation of the endogenous adenylate cyclase. During the
hybrid-depletion screening for Ca^{2+} receptor clones,
oocytes injected with hybrid depleted mRNA are assayed for
isoproterenol-induced adenylate cyclase activation. A
positive response in this assay serves to indicate that any
observed inhibition of Ca^{2+} receptor response is specific,
and not due to a general inhibition of the total mRNA
population.

The hybrid-depletion screening strategy can result in
the isolation of clones that do not contain a complete
protein coding region. Positive clones isolated by this
screening strategy are sequenced to determine their protein
coding capacity. Northern blot analysis of human
parathyroid gland RNA permits the determination of the size
of the complete mRNA corresponding to specific clones. If
positive clones do not appear to be full length, the cloned
cDNA will be used as a hybridization probe to screen a
parathyroid gland cDNA library for complete cDNAs.

A variety of cell lines are capable of coupling
exogenously expressed receptors to endogenous functional
responses. A number of these cell lines (e.g., NIH-3T3,
HeLa, NG115, CHO, HEK 293 and COS7) can be tested to confirm
that they lack an endogenous Ca^{2+} receptor. Those lines
lacking a response to external Ca^{2+} can be used to

establish stably transfected cell lines expressing the
cloned Ca^{2+} receptor.

Production of these stable tansfectants is
accomplished by transfection of an appropriate cell line
with a eukaryotic expression vector, such as pMSG, in which
the coding sequence for the Ca^{2+} receptor cDNA has been
cloned into the multiple cloning site. These expression
vectors contain a promoter region, such as the mouse mammary
tumor virus promoter (MMTV), that drive high-level
transcription of cDNAs in a variety of mammalian cells. In
addition, these vectors contain genes for the selection of
cells that stably express the cDNA of interest. The
selectable marker in the pMSG vector encodes an enzyme,
xanthine-guanine phosphoribosyl transferase (XGPRT), that
confers resistance to a metabolic inhibitor that is added to
the culture to kill the nontransfected cells. A variety of
expression vectors and selection schemes are usually
assessed to determine the optimal conditions for the
production of Ca^{2+} receptor expressing cell lines for use
in high throughput screening assays.

The most effective method for transfection of
eukaryotic cell lines with plasmid DNA varies with the given
cell type. The Ca^{2+} receptor expression construct will be
introduced into cultured cells by the appropriate technique,
either Ca^{2+} phosphate precipitation, DEAE-dextran
transfection, lipofection or electroporation.

Cells that have stably incorporated the transfected
DNA will be identified by their resistance to selection
media, as described above, and clonal cell lines will be
produced by expansion of resistant colonies. The expression

of the Ca^{2+} receptor cDNA by these cell lines will be
assessed by solution hybridization and Northern blot
analysis. Functional expression of the receptor protein
will be determined by measuring the mobilization of
intracellular Ca^{2+} in response to externally applied
Ca^{2+} receptor agonists.

Cloning the Ca^{2+} receptor enables both structural
and functional studies of this novel receptor.
Recombinantly produced receptor may be crystallized for
structural studies. Stably transfected cell lines
expressing the receptor can be used for high-throughput
screening of natural product or other compound libraries.
Molecules of the requisite potency and specificity can be
labeled (radioactively or fluorescently). The ability of
test molecules/extracts to displace such a labeled molecule
will form the basis of a high-throughput assay for screening.

Another strategy for cloning an inorganic-ion
receptor in oocytes is as follows.

The general procedures for isolation, defolliculation
and injection of frog oocytes are described below. In brief,
Xenopus laevis frogs are anesthetized by immersion in 0.17%
Tricaine and a lobe of the ovary removed and minced into
small pieces. Individual stage V-VI oocytes are isolated
using an L-shaped capillary tube after incubating the
ovarian tissue in a calcium-free buffer at room temperature
for 90 min. The separated oocytes [usually 200-300 per
experiment] are then incubated in collagenase solution
[Sigma, Type II; 2 mg/ml] for an additional 90 min following
which the follicular layer can be removed with fine
forceps. The defolliculated oocytes are incubated overnight

in frog ringer [ND96] at 17°C and degenerating oocytes
removed [usually <2-3% of the total]. Surviving oocytes are
then injected with 50 nl H_2O [control] or
poly(A)$^+$RNA[15-50 ng/oocyte; in 50 nl H_2O] and then
incubated for 2-4 days at 17°C. (The frog ringer, ND96,
contains (mM): NaCl (96), KCl (2), $CaCl_2$ (0.5), $MgCl_2$
(0.5), HEPES (5), pyruvate (2.5)].

Total RNA is extracted from the tissue [kidney,
osteoclasts, etc.] with guanidinium thiocyanate and
separated on a CsCl cushion. Poly(A)$^+$RNA is then isolated
by oligo(dT) cellulose chromatography [two passes through
the column]. The integrity of the poly(A)$^+$RNA is assessed
by agarose gel electrophoresis. The ability of 25-50 ng of
poly(A)$^+$RNA to give rise to Ca^{2+}-dependent Cl^- channel
activity upon exposure to extracellular Gd^{3+} is assessed
[i.e., as evidence for Ca^{2+} receptor activity]. The next
step is to localize the mRNA expressing Ca^{2+} receptor
activity to a small size range (~1 kb). For this purpose,
~100 µg of poly(A)$^+$RNA is separated by sucrose
gradient and 40 size fractions collected from the gradient.
Alternatively, ~200-300 µg of poly(A)$^+$RNA can be
fractionated by preparative, continuous flow agarose gel
electrophoresis [Hediger, M.A.: Anal. Biochem. 159:280-86,
(1986) and 70-90 fractions collected. X. laevis oocytes are
injected with pools of fractions [3-5 fractions/pool] of
poly(A)+RNA [0.2-0.5 ng/oocyte] fractions from the pool(s)
and the ability of these pools to give rise to Ca^{2+}
receptor activity assessed. Individual fractions from the
pool(s) giving the highest Ca^{2+} receptor activity are
injected into oocytes to define the fraction(s) within this

pool of poly(A)+RNA that give the highest expression of the
Ca^{2+} receptor in oocytes.

A directional cDNA library is constructed from this
poly(A)+RNA pool using the SuperScript Plasmid system
[pSPORT1; BRL]. cDNA is generated using the SuperScript,
MuMLV-RT, reverse transcripts [many of which will be
full-length for the coding region]. cDNA is sized by gel
electrophoresis and the appropriate cDNA size region [based
on the poly(A)+RNA size range] is extracted using GENECLEAN
II [Bio 101]. This size selected cDNA is then directionally
ligated into the pSPORT1 plasmid vector using a Not I primer
at the 3' end and an adapter for Sal I at the 5' end. The
resulting plasmids are introduced into ELECTROMAX DH10B
cells [BRL] by electroporation. The transformed bacteria
are grown on nitrocellulose filters [500-800
colonies/filter] and master filters stored at 4°C
[short-term] or -70°C [long-term]. Replicate filters are
grown and plasmid DNA is isolated from the filters,
linearized by restriction cutting, and then used as a
template for sense cRNA synthesis by in vitro transcription
[T7 promoter in the presence of Cap-analog]. The pools of
cRNA are individually injected into oocytes which are
assayed for expression of Gd^{3+}-induced [1-100μM]
activation of Cl^- currents. A standard sib selection
procedure is utilized to identify a clone that expresses
Ca^{2+} receptor activity in oocytes.

A restriction map of the Ca^{2+} receptor cDNA is
generated and suitable restriction fragments subcloned into
pSPORT1. Subcloned cDNAs are bidrectionally sequenced using
standard methods [Sequenase Polymerase 2® 2, USB].

Sequencing primers (18-mer) are used where necessary to sequence regions within or between subclones or to resolve compressions or ambiguous regions of sequence. The coding region for the Ca^{2+} receptor protein is determined from the longest open reading frame that has a start site preferably homologous with the Kozak consensus sequence. Hydropathy and other protein algorithms are used to generate a topology for the Ca^{2+} receptor protein. The nucleotide sequence of the cDNA, the amino acid sequence of the Ca^{2+} receptor protein, and the protein topology are compared with that for the other Ca^{2+} receptors and other known cDNAs and proteins present in the database. Homologous regions might represent domains involved in cation binding or regulation.

The presently preferred method for isolating inorganic-ion receptor nucleic acid is based upon hybridization screening.

Region specific primers or probes derived from BoPCaR 1 can be used to prime DNA synthesis and PCR amplification, as well as to identify colonies containing cloned DNA encoding a member of the inorganic ion receptor family using known methods (Innis et al., PCR Protocols, Academic Press, San Diego, CA (1990)).

When using primers that are derived from nucleic acid encoding an inorganic-ion receptor, one skilled in the art will recognize that by employing high stringency conditions, annealing at 50-60°C, sequences which are greater than about 76% homologous to the primer will be amplified. By employing lower stringency conditions, annealing at 35-37°C, sequences which are greater than about 40-50% homologous to

- 93 -

the primer will be amplified.

When using DNA probes derived from inorganic ion
receptors for colony/plaque hybridization, one skilled in
the art will recognize that by employing high stringency
condition, hybridization at 50-65°C, 5X SSPC, 50% formamide,
wash at 50-65°C, 0.5X SSPC, sequences having regions which
are greater than about 90% homologous to the probe can be
obtained, and by employing lower stringency conditions,
hybridization at 35-37.°C, 5X SSPC, 40-45% formamide, wash at
42°C SSPC, sequences having regions which are greater than
35-45% homologous to the probe will be obtained.

Any tissue can be used as the source for the genomic
DNA encoding members of the inorganic-ion receptor family.
However, with respect to RNA, the most preferred source is
tissues which express elevated levels of the desired
inorganic-ion receptor family member. In the present
invention, oocyte injection and two-electrode whole oocyte
voltage clamping was used to identify expression from such a
tissue source. However, using the sequences disclosed
herein, it is now possible to identify such cells using the
inorganic-ion receptor sequence as a probe in northern blot
or in situ hybridization procedures, thus eliminating the
necessity of the procedures employed to clone the first
member of this family and eliminating the need to obtain RNA
from a tissue which expresses elevated levels of
inorganic-ion receptor.

The present invention further provides methods of
identifying cells or tissues which express a member of the
inorganic-ion receptor family. A probe comprising the DNA
sequence, for example, of BoPCaR 1, a fragment thereof, or a

- 94 -

DNA sequence encoding another member of the inorganic-ion receptor family of proteins can be used as a probe or amplification primer to detect cells which express a message homologous to the probe or primer. One skilled in the art can readily adapt currently available nucleic acid amplification or detection techniques so that it employs probes or primers based on the sequences encoding a member of the inorganic-ion receptor family.

Given the appropriate cells or tissues expressing other receptors, these receptors may be cloned in a manner analogous to that described above for the parathyroid cell calcium receptor. For example, mRNA from human osteoclastoma tissue encodes the osteoclast calcium receptor (Figure 34). Thus, to isolate a clone for the human osteoclast receptor, one need only isolate mRNA from osteoclastoma tissue, prepared a cDNA library and assay/fractionate subpools as described above. Furthermore, the preferred receptors for drug screening are of human origin. A clone encoding a receptor from one species may be used to obtain the corresponding human cDNA clone by cross-hybridization as is well known by those skilled in the art. In addition, the clone of the parathyroid cell or other cell receptor allows isolation of genes encoding similar inorganic-ion sensing proteins in other cells, and expression of those proteins. This is achieved by a variety of approaches. Southern blot analysis of human genomic DNA, utilizing the Ca^{2+} receptor cDNA as a hybridization probe, will give an indication of the number of related sequences encoded within the genome; hybridization at varying stringencies will give an indication of the degree of

divergence among the related sequences. This will provide
information about the potential number of genes encoding
related receptor proteins. Northern blot analysis with
Ca^{2+} receptor cDNA as probe will determine if the same or
related transcripts are present in various tissues. If
related transcripts homologous to the parathyroid cell
Ca^{2+} receptor are detected, it is a relatively simple
matter to obtain clones of these mRNAs, either by screening
the appropriate cDNA libraries or by polymerase chain
reaction (PCR) techniques.

Targeted gene walking (TGW) is a modification of a
standard polymerase chain reaction (PCR) that allows
amplification of unknown DNA sequences adjacent to short
segments of known sequence. Parker et al., Nucl. Acids
Res., 19:3055, (1991). Unlike conventional PCR techniques
that amplify DNA sequences between two known primer sites,
TGW can amplify DNA adjacent to one such site. Thus, TGW
can serve as a replacement for conventional cloning and
library screening methods for isolating sequences upstream
or downstream from known sequences. The procedure can be
used to isolate genes from any starting DNA template for
which a limited amount of sequence information is known.

First, several standard PCR reactions are run in
parallel using one "targeted primer" and different "walking
primers." The targeted primer is a sequence-specific primer
exactly complementary to a known sequence on the DNA
molecule of interest, and is directed towards unknown
adjacent sequences. The walking primers are non-specific
sequences not complementary to DNA near the target primer.
The walking primers can be any oligonucleotides unrelated to

- 96 -

the target primer sequence. In the first series of PCRs,
products are produced only when a walking primer anneals to
a DNA strand contiguous with and complementary to the strand
to which the targeted primer has hybridized. The PCR
products of interest are preferably within the 5 kilobase
size range. Amplification products are produced with as
many as 60% mismatched nucleotides within the walking primer
relative to DNA template. Perfect base-pairing is required
only for the first two 3' nucleotides of the walking primer,
but partial homology is tolerated otherwise. Annealing
temperature is a key variable in determining the number of
PCR products, as identified by agarose gel electrophoresis.

Second, an oligomer extension assay is performed
using an "internal detection primer." This primer
represents known sequences between the previous two primers,
contiguous with the targeted primer. The internal detection
primer is kinased with ^{32}P-gamma-ATP, then used in a
single PCR cycle with DNA from the first PCR as template.
This extension identifies products in the first PCR
contiguous with the targeted primer. These new products are
identified by agarose gel electrophoresis and
autoradiography. Any products that do no hybridize to the
internal detection primer represent non-contiguous
amplification products produced by any subset of the
primers.

Last, bands identified in the oligomer extension
assay are excised from the gel, and reamplified by standard
PCR using target primer and the walking primer that produced
the band initially. This new PCR band is then sequenced
directly to provide previously unknown sequence

information.

        To extend information in the opposite direction,
complements are made of the targeted and internal detection
primers, and their order is reversed in the protocol.

        In performing hybridization screening and cloning,
the following should be considered. Since there is
degeneracy in the genetic code, more than one codon exists
for almost all the amino acids (except tryptophan and
methionine). Moreover, the frequency of usage of any
particular codon is different in non-humans as compared to
humans. Taking into account codon degeneracy and
human-preferred codons, oligonucleotides are synthesized.
Moreover, oligonucleotides with various permutations of
possible codons are also synthesized. To avoid an excessive
number, not all possible sequences resulting from the
degeneracy of the code need be synthesized. Rather, a
subset of codons are chosen which have the highest frequency
of occurrence.

        Novel receptor clones so obtained can be assessed
functionally by expression, either in oocytes or in
transfected cell lines. Transfected cell lines expressing a
cell-specific inorganic-ion receptor can then provide a
means of high-throughput screening for molecules that act
specifically on the ion-sensing mechanism of, for example,
osteoclasts or juxtaglomerular cells.

        In an alternative method, the calcium receptor can be
cloned by expression in eukaryotic cells. For example, a
cDNA library can be prepared from parathyroid mRNA and
cloned into a eukaryotic expression vector such as, pCDNA1.
Subpools from this library can be transfected into

eukaryotic cells such as COS7 or HEK293 cells resulting in
relatively high-level transient expression of encoded cDNA
sequences. Cells transfected with a function calcium
receptor clone will express the calcium receptor which can
then be activated by calcium, neomycin or other calcimimetic
compounds. If cells are first loaded with a fluorometric
indicator for $[Ca^{2+}]_i$, activation of the calcium
receptor results in increased fluorescence. Thus library
subpools containing the calcium receptor are identified by
their ability, upon transfection into eukaryotic cells, to
induce a calcium or calcimimetic-specific increase in
fluorescence. This fluorescence can be detected using
either a fluorimeter or a fluorescence-activated cell sorter
(FACS).

        We have also developed a "calcium trapping assay" for
the detection of COS 7 cells expressing G-protein coupled
receptors. In this assay COS 7 cell monolayers are
transfected with cDNA clones from a bovine parathyroid cDNA
library (e.g., subfractions or pools from a library prepared
in pCDNA1) and are assayed for their ability to trap
radioactive $^{45}Ca^{++}$ in response to treatment with an
agonist for the Ca^{2+} receptor. The monolayers undergo
emulsion autoradiography and cells that have trapped
$^{45}Ca^{++}$ are identified by the presence of photographic
grain clusters under dark-field microscopy. Library pools
that produce a positive signal are then sequentially
subdivided until a single cDNA that produces the signal is
identified.

        The calcium receptors appear to be functionally
related to a class of receptors which utilize so-called "G"

proteins to couple ligand binding to intracellular signals.
Such "G-coupled" receptors may elicit increases in
intracellular cyclic AMP due to the stimulation of adenylyl
cyclase by a receptor activated "G_s" protein, or else may
elicit a decrease in cyclic AMP due to inhibition of
adenylyl cyclase by a receptor activated "G_i" protein.
Other receptor activated G proteins elicit changes in
inositol trisphosphate levels resulting in release of Ca^{++}
from intracellular stores. This latter mechanism is
particularly pertinent to calcium receptors. All known
G-coupled receptors are structurally related, having seven
conserved transmembrane domains. A number of such receptors
have been cloned based on sequence homology to previously
cloned receptors. One particularly useful approach is to
employ degenerate primers homologous to the conserved
transmembrane domain coding regions and to amplify DNA
regions encoding these sequences using polymerase chain
reaction (PCR). Thus, such oligonucleotide primers are
mixed with genomic DNA or cDNA to RNA isolated from the
tissue of choice and PCR carried out. Some experimentation
may be required to specifically amplify novel G-coupled
receptor sequences from the tissue of choice since these are
not necessarily identical to already known G-coupled
receptors but, this is well understood by those skilled in
the art [see for example, Buck, L. and Axel, R. (1991) Cell,
65:175-187].

        Calcium receptors may also be cloned by such a PCR
approach. Two examples of degenerate oligonucleotide primer
pairs which can be used to PCR amplify sequences encoding
calcium receptors are given below. The first pair is based

on the cross-homology exhibited by the majority of G-coupled
receptors in the sequences encoding transmembrane domains II
and VII respectively. The second primer pair is based on
the cross-homology exhibited by a more divergent subgroup of
G-coupled receptors which includes the calcitonin, secretin,
PTH and GLP receptors. This pair corresponds to conserved
sequences encoding transmembrane domains III and VII. When
one or both of these two primer pairs are mixed with cDNA
from parathyroid or osteoclast tissues, for example, PCR
amplification results in the generation of amplified DNAs of
about 500 to 800 base pairs. These DNAs can be isolated and
analyzed for the presence of calcium receptor sequences.
For example, each amplified sequence can be used as a probe
to isolate a full-length cDNA clone which can then be
assessed in one or more of the assays indicated above to
determine if an inorganic-ion receptor is encoded.

PRIMER PAIR 1:

```
5'   GACTACTTCCTGGTGAACCTGGC  3'
     A    T    T T C C    C
_____

5'   TCAAIGAGCCIGTAGATGATGGGGTT   3'
       T C    AGG A  A AC CA  A
                     GC
```

PRIMER PAIR 2:

```
5'  AACTACTICTGGITGCTGGTGGAGGGCCTCTA   3'
                  C         A  GG G
                  T
_____

5'  GAAGCAGTAIATAATGGCAACAAAAAAGCCCTGGAA 3'
      A       G CA     G    GA
                       C    T
```

        In an alternative method, the inorganic-ion
receptor can be cloned by use of a monoclonal antibody

generated against the receptor. Monoclonal antibodies
provide powerful tools for the immunoaffinity purification
of specific proteins. Once purified, limited amino acid
sequence data can be obtained from the protein of interest,
and used to design oligonucleotide sequence probes to screen
for clones of the complete cDNA sequence.

An example involving a calcium receptor will be
described. For production of hybridomas, whole bovine
parathyroid gland cells are used as the immunogen.
Purified, dispersed cells are obtained, and live or fixed
cell preparations are injected intraperitoneally into the
appropriate mouse strain, according to established
procedures. Standard protocols are followed for
immunization schedules and for the production of
hybridomas. A two-step screening procedure is used to
identify hybridomas secreting monoclonal antibodies that
recognize the Ca^{2+} receptor. The initial screen will
identify those monoclonals that recognize parathyroid cell
surface antigens. Immunohistochemical techniques are then
used to screen hybridoma supernatants for the presence of
mouse antibodies that bind to the surface of parathyroid
cells. This screen can be performed on fixed sections of
parathyroid gland tissue, or on dispersed cells in primary
culture. The techniques for this assay are well established
in the literature.

This screen will identify hybridomas producing
monoclonal antibodies to a variety of cell-surface
determinants, and monoclonals specific for the Ca^{2+}
receptor would be expected to comprise only a small subset
of these. To identify monoclonal antibodies that bind to

- 102 -

the Ca^{2+} receptor, hybridoma supernatants that test
positive in the initial screen are assayed for their ability
to block the response of cultured parathyroid cells to
Ca^{2+} receptor agonists. Some antibodies that bind to the
extracellular domain of the receptor are expected to inhibit
or activate ligand binding or to otherwise interfere with or
affect receptor activation.

Monoclonal antibodies positive in both screens
are characterized through Western blotting,
immunoprecipitation and immunohistochemistry. This permits
the determination of the size of the antigen that is
recognized and its tissue distribution. The appropriate
monoclonal antibody is then used for purification of the
Ca^{2+} receptor protein by immunoaffinity chromatography,
following standard techniques. Sufficient quantities of
protein are obtained to allow limited amino acid sequence
determination. Degenerate oligonucleotide probes are then
designed on the basis of the peptide sequence information.
These probes are then used to screen parathyroid gland cDNA
libraries for full-length clones of the Ca^{2+} receptor.
Clones obtained are characterized by DNA sequencing and by
functional expression in the oocyte system and in cultured
mammalian cell lines.

Alternatively, the antibodies can be used to
screen expression libraries, e.g., cDNA libraries in λgt11
or its equivalent, to determine those clones expressing
antigenically reactive protein. Such clones can then be
sequenced to determine whether they encode a protein that
might be a Ca^{2+} receptor.

It will also be appreciated by those skilled in

the art that phage display libraries can be used to clone
and analyze calcium receptors in place of monoclonal
antibodies. In these libraries, antibody-variable regions or
random peptides are shotgun cloned into phage expression
vectors such that the antibody regions or peptides are
displayed on the surface of the phage particle. Phage(s)
which display antibody regions or peptides capable of high
specific binding to calcium receptors will bind to cells
which display these receptors (e.g., parathyroid cells,
C-cells, osteoclasts, etc.). Hundreds of millions of such
phage can be panned against these cell types preferentially
selecting those phage which can bind to these cells (which
includes those phage binding to calcium receptors). In this
manner, the complexity of the library can be vastly
reduced. Iterative repetition of this process results in a
pool of phage which bind to the cell type used.
Subsequently, the screens described above for monoclonal
antibodies can be used to isolate phage which display
calcium receptor-binding antibody or peptide regions, and
these phage can be used to isolate the calcium receptor for
purposes of structural identification and cloning. Kits to
prepare such phage- display libraries are commercially
available (e.g., Stratacyte, or Cambridge Antibody
Technology Limited). Recombinant phage endowed with such
calcium receptor-binding properties can also be used in lieu
of monoclonal antibodies in the various analyses of calcium
receptors. Such phage can also be used in high throughput
binding-competition screens to identify organic compounds
capable of functional binding to calcium receptors which can
serve as structural leads for the development of human

therapeutics acting at the calcium receptor.

In another alternative, affinity cross-linking
of radioligands to their receptors can be used to isolate
the receptor protein as described by Pilch & Czech, 1
Receptor Biochem. Methodol. 161, 1984. Covalent attachment
of a radioligand allows extensive washing to remove
non-specific binding. For example, a high- affinity
molecule, e.g., a random copolymer of arginine and tyrosine
(MW = 22K; argtyr ratio = 4:1) which mobilizes intracellular
Ca^{2+} with an EC_{50} of about 100 nM or less, is iodinated
with ^{125}I, and cross-linked. Protamines, because of their
much smaller size, may be preferable in cross-linking
studies and can be reductively alkylated as described by
Dottavio-Martin & Ravel, 87 Analyt. Biochem. 562, 1978.

Nonspecific labelling is kept to a minimum by
cross-linking in the presence of unlabeled polycations and
di- and trivalent cations. At high concentrations of these
molecules nonspecific interactions of the label with the
cell surface might be reduced.

The invention provides isolated nucleic acid
sequences encoding inorganic-ion receptors and the isolated
receptors themselves. It also provides unique fragments of
the foregoing. The term "isolated" refers to a nucleic acid
sequence: (i) amplified in vitro by, for example, polymerase
chain reaction (PCR); (ii) synthesized by, for example,
chemical synthesis; (iii) recombinantly produced by cloning;
or (iv) purified, as by cleavage and gel separation. The
term "isolated" when used in descriptions of polypeptide or
amino acid sequences refers to polypeptides resulting from
an expression system using the isolated nucleic acid

- 105 -

sequences of the invention, as well as polypeptides
synthesized by, for example, chemical synthetic methods, and
polypeptides separated from native biological materials, and
then substantially purified using conventional protein
analytical procedures.

Also included within the scope of the invention
are unique fragments of inorganic-ion receptors. The term
unique fragments refers to portions of the receptor that
find no counterpart in known sequences as of the date of
this invention. These polypeptide fragments can be readily
identified as unique by scanning protein databases known as
of the time of the invention for identical peptides. In
addition to generating fragments of receptors from
expression of cloned partial sequences of receptor DNA,
fragments can be generated directly from the intact
protein. Proteins are specifically cleaved by proteolytic
enzymes, including, but not limited to, trypsin,
chymotrypsin or pepsin. Each of these enzymes is specific
for the type of peptide bond it attacks. Trypsin catalyzes
the hydrolysis of peptide bonds whose carbonyl group is from
a basic amino acid, usually arginine or lysine. Pepsin and
chymotrypsin catalyze the hydrolysis of peptide bonds from
aromatic amino acids, particularly tryptophan, tyrosine and
phenylalanine. Alternate sets of cleaved polypeptide
fragments are generated by preventing cleavage at a site
which is susceptible to a proteolytic enzyme. For example,
reaction of the ε-amino groups of lysine with
ethyltrifluorothioacetate in mildly basic solution yields a
blocked amino acid residue whose adjacent peptide bond is no
longer susceptible to hydrolysis by trypsin. Goldberger et

al. Biochem., 1:401 (1962). Treatment of such a polypeptide
with trypsin thus cleaves only at the arginyl residues.
Polypeptides also can be modified to create peptide linkages
that are susceptible to proteolytic enzyme catalyzed
hydrolysis. For example, alkylation of cysteine residues
with ß-haloethylamines yields peptide linkages that are
hydrolyzed by trypsin. Lindley, Nature, 178: 647 (1956).
In addition, chemical reagents that cleave polypeptide
chains at specific residues can be used. Witcop, Adv.
Protein Chem. 16: 221 (1961). For example, cyanogen bromide
cleaves polypeptides at methionine residues. Gross &
Witkip, J. Am Chem Soc., 83: 1510 (1961). Thus, by treating
an inorganic ion receptor, such as, for example, a human
calcium receptor or fragments thereof, with various
combinations of modifiers, proteolytic enzymes and/or
chemical reagents, numerous discrete overlapping peptides of
varying sizes are generated. These peptide fragments can be
isolated and purified from such digests by chromatographic
methods.

        Alternatively, fragments can be synthesized
using an appropriate solid state synthetic procedure.
Steward and Young, Solid Phase Peptide Synthesis,
Freemantle, San Fansisco, CA (1968).

        The fragments may be used in assays, as
ion-binding agents, in the production of antibodies and the
like. The fragments also may be selected to have desirable
biological activities. For example, the fragment may
include just the binding site, or a site which binds to
agonists or antagonists (e.g. of calcium), as described
herein. Such fragments are readily identified by

those of ordinary skill in the art using routine methods to
detect specific binding to the fragment. For example, in
the case of a calcium receptor, a fragment to be tested can
be expressed, using recombinant DNA methodology, from a
fragment of the gene encoding the recombinant receptor.
This fragment is then contacted with calcium or another
chemical under appropriate association conditions to
determine whether the calcium binds to the fragment. These
fragments are useful in screening assays for agonists and
antagonists of calcium, and for therapeutic effect where it
is useful to remove calcium from serum, or other bodily
tissues.

     Other useful fragments include those that have
only the external portion, membrane-spanning portion, or
intracellular portion of the receptor. These portions are
readily identified by comparison of the amino acid sequence
of the receptor with those of known receptors, or by other
standard methodology. These fragments are useful for
forming chimeric receptors with fragments of other receptors
so that a cell lacking a receptor may be formed with an
intracellular portion which performs a desired function
within that cell, and an extracellular portion which causes
that cell to respond to the presence of ions, or those
agonists or antagonists described herein. Such chimeric
receptor genes when appropriately formulated are useful
genetic therapies for a variety of diseases involving
dysfunction of receptors or where modulation of receptor
function provides a desirable effect in the patient. In
addition, chimeric receptors can be constructed such that
the intracellular domain is coupled to a desired enzymatic

process which can be readily detected by colorometric,
radiometric luminometric, spectrophotometric or fluorometric
assays and is activated by interaction of the extracellular
portion with its native ligand (e.g. calcium) or agonists
and or antagonists of the invention. Cells expressing such
chimeric receptors are the basis for facilitated screens for
the discovery of new agonists and/or antagonists of the
invention.

The invention also features muteins or analogs
and other derivatives of isolated receptors, as described
herein. Thus, the invention embraces not only naturally
occurring proteins, but also such derivatives. Such
derivatives have the desired receptor activity described
herein and are generally identified by methods described
herein. While examples of such proteins, and genes encoding
them, are provided, these examples are not limiting in the
invention but demonstrate only the variation that can be
associated with such genes and proteins. Generally, the
gene will have the amino acid sequence of the native
receptor, but may be altered at one or more amino acid
positions which do not significantly affect the receptor
activity of the produced receptor protein. Thus, for
example, in non-conserved regions of the calcium receptor
protein (i.e., those regions not necessary for receptor
activity) amino acids may be deleted, added or substituted.
In more conserved regions, which are required for receptor
activity, amino acids may be more conservatively
substituted. For example, one or more amino acid residues
within the sequence can be substituted by another amino acid
of a similar polarity which acts as a functional
equivalent. Substitutes for an amino acid within the

sequence may be selected from other members of the class to
which the amino acid belongs. The non-polar (hydrophobic)
amino acids include alanine, leucine, isoleucine, valine,
proline, phenylalanine, tryptophan and methionine. The
polar neutral amino acids include glycine, serine,
threonine, cysteine, tyrosine, asparagine, and glutamine.
The positively charged (basic) amino acids include arginine,
lysine and histidine. The negatively charged (acidic) amino
acids include asparatic acid and glutamic acid.

          Those of ordinary skill in the art will
recognize that standard procedures can be used to determine
the conserved and non-conserved regions of the protein using
in vitro mutagenesis techniques or deletion analyses, and
that all such derivatives are equivalent of those described
below.

          Also included within the scope of the invention
are receptor proteins or unique fragments or derivatives
thereof which are differentially modified during or after
translation, e.g., by phosphorylation, glycosylation,
crosslinking, acylation, proteolytic cleavage, linkage to an
antibody molecule, membrane molecule or other ligand,
(Ferguson et al., 1988, Ann. Rev. Biochem. 57:285-320).

          In addition, recombinant nucleic acid sequences
of the invention may be engineered so as to modify
processing or expression of receptor sequences. For
example, and not by way of limitation, the coding sequence
may be combined with a promoter sequence and/or a ribosome
binding site using well characterized methods, and thereby
facilitate improve expression and bioavailability.

          Additionally, a given recombinant coding

sequence can be mutated in vitro or in vivo, to create
variations in coding regions and/or form new restriction
endonuclease sites or destroy preexisting ones, to
facilitate further in vitro modification. Any technique for
mutagenesis known in the art can be used including, but not
limited to, in vitro site-directed mutagenesis (Hutchinson,
et al., 1978, J. Biol. Chem. 253:6551), use of TAB®
linkers (Pharmacia), PCR-directed mutagenesis, and the like.

In addition, codons may be modified such that
while they encode an identical amino acid, that codon may be
a preferred codon in the chosen expression system.

The invention also provides unique fragments of
nucleic acid encoding an inorganic-ion receptor. The term
"unique fragments" refers to portions of the nucleic acid
that find no identical counterpart in known sequences as of
the date of this invention. These fragments can be
identified easily by an analysis of nucleic acid data bases
existing as of the date of the invention to detect
counterparts. Unique fragments are useful inter alia in
cloning procedures and assays.

The invention further provides receptor binding
agents including antibodies and/or fragments thereof which
can be conjugated to a toxin moiety, or expressed along with
a toxin moiety as a recombinant fusion protein. The toxin
moiety will bind to and enter a target cell using
interaction of the binding agent and the corresponding
target cell surface receptor. The toxic moiety to which the
agent, antibody and/or fragment is conjugated can be a
protein such as, for example, pokeweed anti-viral protein,
ricin, gelonin, abrin, diphtheria exotoxin, or Pseudomonas

exotoxin. The toxin moiety can also be a high
energy-emitting radionuclide such as cobalt-60.   The
chemical structure of the toxin moiety is not intended to
limit the scope of the invention in any way.   Those of
ordinary skill in the art will recognize that a large
variety of possible moieties can be linked to the binding
agents. See, for example, "Conjugate Vaccines",
Contributions to Microbiology and Immunology, J.M. Cruse and
R.E. Lewis, Jr (eds). Carger Press, New York, (1989).   It
further is art recognized that many toxin moieties,
including those specifically listed above, are considered
pharmaceutically acceptable.

        The conjugation of the binding agent to another
moiety (e.g. bacterial toxin) can be accomplished by any
chemical reaction that will bind the two molecules so long
as both molecules retain their respective activity. This
linkage can include many chemical mechanisms, for instance
covalent binding, affinity binding, intercalation,
coordinate binding and complexation. The preferred binding
is, however, covalent binding. The covalent binding can be
achieved either by direct condensation of existing side
chains or by the incorporation of external bridging
molecules. Many bivalent or polyvalent linking agents are
useful in coupling protein molecules, such as an antibody,
to other molecules. For example, representative coupling
agents can include organic compounds such as thioesters,
carbodiimides, succinimide esters, diisocyanates,
glutaraldehydes, diazobenzenes and hexamethylene diamines.
This listing is not intended to be exhaustive of the various
classes of coupling agents known in the art but, rather, is

exemplary of the more common coupling agents. (See Killen and Lindstrom 1984, "Specific killing of lymphocytes that cause experimental Autoimmune Myasthenia Gravis by toxin-acetylcholine receptor conjugates." Jour. Immun. 133:1335-2549; Jansen, F.K., H.E. Blythman, D. Carriere, P. Casella, O. Gros, P. Gros, J.C. Laurent, F. Paolucci, B. Pau, P. Poncelet, G. Richer, H. Vidal, and G.A. Voisin. 1982. "Immunotoxins: Hybrid molecules combining high specificity and potent cytotoxicity". Immunological Reviews 62:185-216; and Vitetta et al., supra).

Using recombinant DNA techniques, the present invention provides for target cells including mammalian target cells which are engineered to express inorganic-ion receptors. For example, the genes for such receptors, cloned according to the methods set forth above, may be inserted into cells which naturally express the receptors such that the recombinant gene is expressed at much higher levels.

The present invention also provides for experimental model systems for studying the physiological role of the receptors. In these model systems, the inorganic-ion receptors, fragments, or derivatives thereof, may be either supplied to the system or produced within the system. Such model systems could be used to study the effects or cell function of receptor excess or depletion. The experimental model systems may be used to study the effects in cell or tissue cultures, in whole animals, or in particular cells or tissues within whole animals or tissue culture systems, or over specified time intervals (including during embryogenesis). A preferred embodiment is assays involving the cloned receptors or parts thereof, including

particularly those useful in high through-put drug screening assays.

In additional embodiments of the invention, a recombinant gene may be used to inactivate the endogenous gene by homologous recombination, and thereby create an inorganic-ion receptor deficient cell, tissue, or animal. For example, and not by way of limitation, a recombinant gene may be engineered to contain an insertional mutation (e.g.. the neo gene) which, when inserted into the genome of a recipient cell, tissue or animal, inactivates transcription of the receptor. Such a construct may be introduced into a cell, such as an embryonic stem cell, by a technique such as transfection, transduction, injection, etc. Stem cells lacking an intact receptor sequence may generate transgenic animals deficient in the receptor.

A "transgenic animal" is an animal having cells that contain DNA which has been artificially inserted into a cell, which DNA becomes part of the genome of the animal which develops from that cell. Preferred transgenic animals are primates, mice, rats, cows, pigs, horses, goats, sheep, dogs and cats. The transgenic DNA may encode for human inorganic-ion receptors. In a further embodiment of the invention, native expression in an animal may be reduced by providing an amount of anti-sense RNA or DNA effective to reduce expression of the receptor.

A variety of methods are available for the production of transgenic animals associated with this invention. DNA can be injected into the pronucleus of a fertilized egg before fusion of the male and female pronuclei, or injected into the nucleus of an embryonic cell

(e.g., the nucleus of a two-cell embryo) following the initiation of cell division (Brinster et al., Proc. Nat. Acad. Sci. USA, 82: 4438-4442 (1985)). Embryos can be infected with viruses, especially retroviruses, modified to carry inorganic-ion receptor nucleotide sequences of the invention.

Pluripotent stem cells derived from the inner cell mass of the embryo and stabilized in culture can be manipulated in culture to incorporate nucleotide sequences of the invention. A transgenic animal can be produced from such cells through implantation into a blastocyst that is implanted into a foster mother and allowed to come to term.

Animals suitable for transgenic experiments can be obtained from standard commercial sources such as Charles River (Wilmington, MA), Taconic (Germantown, NY), Harlan Sprague Dawley (Indianapolis, IN), etc.

The procedures for manipulation of the rodent embryo and for microinjection of DNA into the pronucleus of the zygote are well known to those of ordinary skill in the art (Hogan et al., supra). Microinjection procedures for fish, amphibian eggs and birds are detailed in Houdebine and Chourrout, Experientia, 47: 897-905 (1991). Other procedures for introduction of DNA into tissues of animals are described in U.S. Patent No., 4,945,050 (Sandford et al., July 30, 1990).

By way of example only, to prepare a transgenic mouse, female mice are induced to superovulate. Females are placed with males, and the mated females are sacrificed by CO_2 asphyxiation or cervical dislocation and embryos are recovered from excised oviducts. Surrounding cumulus cells

are removed.   Pronuclear embryos are then washed and stored
until the time of injection.   Randomly cycling adult female
mice are paired with vasectomized males.   Recipient females
are mated at the same time as donor females.   Embryos then
are transferred surgically.

The procedure for generating transgenic rats is
similar to that of mice.   See Hammer et al., Cell,
63:1099-1112 (1990).

Methods for the culturing of embryonic stem (ES)
cells and the subsequent production of transgenic animals by
the introduction of DNA into ES cells using methods such as
electroporation, calcium phosphate/DNA precipitation and
direct injection also are well known to those of ordinary
skill in the art.   See, for example, Teratocarcinomas and
Embryonic Stem Cells, A Practical Approach, E.J. Robertson,
ed., IRL Press (1987).

In cases involving random gene integration, a
clone containing the sequence(s) of the invention is
co-transfected with a gene encoding resistance.
Alternatively, the gene encoding neomycin resistance is
physically linked to the sequence(s) of the invention.
Transfection and isolation of desired clones are carried out
by any one of several methods well known to those of
ordinary skill in the art (E.J. Robertson, supra).

DNA molecules introduced into ES cells can also
be integrated into the chromosome through the process of
homologous recombination.   Capecchi, Science, 244: 1288-1292
(1989).   Methods for positive selection of the recombination
event (i.e., neo resistance) and dual positive-negative
selection (i.e., neo resistance and gancyclovir resistance)

- 116 -

and the subsequent identification of the desired clones by
PCR have been described by Capecchi, supra and Joyner et
al., Nature, 338: 153-156 (1989), the teachings of which are
incorporated herein. The final phase of the procedure is to
inject targeted ES cells into blastocysts and to transfer
the blastocysts into pseudopregnant females. The resulting
chimeric animals are bred and the offspring are analyzed by
Southern blotting to identify individuals that carry the
transgene.

Procedures for the production of non-rodent
mammals and other animals have been discussed by others.
See Houdebine and Chourrout, supra; Pursel et al., Science
244: 1281-1288 (1989); and Simms et al., Bio/Technology, 6:
179-183 (1988).

Uses

Primary hyperparathyroidism (HPT) is
characterized by hypercalcemia and elevated levels of
circulating PTH. One of the major defects in HPT appears to
be a diminished sensitivity of parathyroid cells to negative
feedback regulation by extracellular Ca^{2+}. Thus, in
tissue from patients with primary HPT, the "set-point" for
extracellular Ca^{2+} is shifted to the right so that higher
than normal concentrations of extracellular Ca^{2+} are
required to depress PTH secretion. Moreover, in primary
HPT, even high concentrations of extracellular Ca^{2+} often
depress PTH secretion only partially. In secondary (uremic)
HPT, a similar increase in the set-point for extracellular
Ca^{2+} is observed even though the degree to which
Ca^{2+} suppresses PTH secretion is normal. The changes in
PTH secretion are paralleled by changes in $[Ca^{2+}]_i$: the

set-point for extracellular Ca^{2+}-induced increases in
$[Ca^{2+}]_i$ is shifted to the right and the magnitude of
such increases is reduced. Moreover, staining of tissue
with a monoclonal antibody that appears to recognize the
Ca^{2+} receptor is diminished in adenomatous and
hyperplastic parathyroid cells.

The Ca^{2+} receptor constitutes a discrete
molecular entity for pharmacological intervention.
Molecules that mimic or antagonize the action of
extracellular Ca^{2+} are beneficial in the long-term
management of both primary and secondary HPT. Such
molecules provide the added impetus required to suppress PTH
secretion which the hypercalcemic condition alone cannot
achieve. Such molecules with greater efficacy than
extracellular Ca^{2+} may overcome the apparent
nonsuppressible component of PTH secretion which is
particularly troublesome in adenomatous tissue.
Alternatively or additionally, such molecules can depress
synthesis of PTH, as prolonged hypercalcemia has been shown
to depress the levels of preproPTH mRNA in bovine and human
adenomatous parathyroid tissue. Prolonged hypercalcemia
also depresses parathyroid cell proliferation _in vitro_, so
calcimimetics can also be effective in limiting the
parathyroid cell hyperplasia characteristic of secondary HPT.

Other cells in the body can respond directly to
physiological changes in the concentration of extracellular
Ca^{2+}. Calcitonin secretion from parafollicular cells in
the thyroid (C-cells) is regulated by changes in the
concentration of extracellular Ca^{2+}. Renin secretion from
juxtaglomerular cells in the kidney, like PTH secretion, is

depressed by increased concentrations of extracellular Ca^{2+}. Extracellular Ca^{2+} causes the mobilization of intracellular Ca^{2+} in these cells. Other kidney cells respond to calcium as follows: elevated Ca^{2+} inhibits formation of $1,25(OH)_2$ Vitamin D by proximal tubular cells, stimulates production of calcium-binding protein in distal tubular cells, and inhibits tubular reabsorption of Ca^{2+} and Mg^{2+} and the action of vasopressin on the medullary thick ascending limb of Henle's loop (MTAL), reduces vasopressin action in the cortical collecting duct cells, and affects vascular smooth muscle cells in blood vessels of the renal glomerulus. Calcium promotes the differentiation of intestinal goblet cells, mammary cells, and skin cells. It also inhibits atrial natriuretic peptide secretion from cardiac atria, reduces cAMP accumulation in platelets, alters gastrin and glucagon secretion, and acts on vascular smooth muscle cells to modify cell secretion of vasoactive factors. Isolated osteoclasts respond to increases in the concentration of extracellular Ca^{2+} with corresponding increases in $[Ca^{2+}]_i$ that arise partly from the mobilization of intracellular Ca^{2+}. Increases in $[Ca^{2+}]_i$ in osteoclasts are associated with an inhibition of functional responses (bone resorption) analogous to PTH secretion in parathyroid cells. Release of alkaline phosphatase from bone-forming osteoblasts is directly stimulated by calcium. Thus, there are sufficient indications to suggest that Ca^{2+}, in addition to its ubiquitous role as an intracellular signal, also functions as an extracellular signal to regulate the responses of certain specialized cells. Molecules of this invention can

be used in the treatment of diseases associated with disrupted Ca^{2+} responses in these cells.

Cloning the Ca^{2+} receptor on parathyroid cells and other cells will allow the presence of homologous proteins in other cells to be directly assessed. A family of structurally homologous Ca^{2+} receptor proteins can thus be obtained. Such receptors will allow understanding of how these cells detect extracellular Ca^{2+} and enable evaluation of the mechanism(s) as a site of action for the therapeutics described herein effective in the treatment of HPT, osteoporosis, and hypertension, and novel therapies for other bone and mineral-related diseases.

Other uses are discussed above. For example, recombinant Ca^{2+} receptor proteins may be used in therapy, and introduced by standard methods, e.q., by transfection of nucleic acid encoding that protein. In addition, such protein is useful in assays for calcimimetic molecules of this invention.

The following examples illustrate the invention but do not limit its scope.

Methods for the culturing of embryonic stem (ES) cells and the subsequent production of transgenic animals by the introduction of DNA into ES cells using methods such as electroporation, calcium phosphate/DNA precipitation and direct injection also are well known to those of ordinary skill in the art. See, for example, Teratocarcinomas and Embryonic Stem Cells, A Practical Approach, E.J. Robertson, ed., IRL Press (1987).

In cases involving random gene integration, a clone containing the sequence(s) of the invention is

co-transfected with a gene encoding resistance.
Alternatively, the gene encoding neomycin resistance is
physically linked to the sequence(s) of the invention.
Transfection and isolation of desired clones are carried out
by any one of several methods well known to those of
ordinary skill in the art (E.J. Robertson, supra).

DNA molecules introduced into ES cells can also
be integrated into the chromosome through the process of
homologous recombination. Capecchi, Science, 244: 1288-1292
(1989). Methods for positive selection of the recombination
event (i.e., neo resistance) and dual positive-negative
selection (i.e., neo resistance and gancyclovir resistance)
and the subsequent identification of the desired clones by
PCR have been described by Capecchi, supra and Joyner et
al., Nature, 338: 153-156 (1989), the teachings of which are
incorporated herein. The final phase of the procedure is to
inject targeted ES cells into blastocysts and to transfer
the blastocysts into pseudopregnant females. The resulting
chimeric animals are bred and the offspring are analyzed by
Southern blotting to identify individuals that carry the
transgene.

Procedures for the production of non-rodent
mammals and other animals have been discussed by others.
See Houdebine and Chourrout, supra; Pursel et al., Science
244: 1281-1288 (1989); and Simms et al., Bio/Technology, 6:
179-183 (1988).

Uses

Primary hyperparathyroidism (HPT) is
characterized by hypercalcemia and elevated levels of
circulating PTH. One of the major defects in HPT appears to

be a diminished sensitivity of parathyroid cells to negative
feedback regulation by extracellular Ca^{2+}. Thus, in
tissue from patients with primary HPT, the "set-point" for
extracellular Ca^{2+} is shifted to the right so that higher
than normal concentrations of extracellular Ca^{2+} are
required to depress PTH secretion. Moreover, in primary
HPT, even high concentrations of extracellular Ca^{2+} often
depress PTH secretion only partially. In secondary (uremic)
HPT, a similar increase in the set-point for extracellular
Ca^{2+} is observed even though the degree to which
Ca^{2+} suppresses PTH secretion is normal. The changes in
PTH secretion are paralleled by changes in $[Ca^{2+}]_i$: the
set-point for extracellular Ca^{2+}-induced increases in
$[Ca^{2+}]_i$ is shifted to the right and the magnitude of
such increases is reduced. Moreover, staining of tissue
with a monoclonal antibody that appears to recognize the
Ca^{2+} receptor is diminished in adenomatous and
hyperplastic parathyroid cells.

The Ca^{2+} receptor constitutes a discrete
molecular entity for pharmacological intervention.
Molecules that mimic or antagonize the action of
extracellular Ca^{2+} are beneficial in the long-term
management of both primary and secondary HPT. Such
molecules provide the added impetus required to suppress PTH
secretion which the hypercalcemic condition alone cannot
achieve. Such molecules with greater efficacy than
extracellular Ca^{2+} may overcome the apparent
nonsuppressible component of PTH secretion which is
particularly troublesome in adenomatous tissue.
Alternatively or additionally, such molecules can depress

synthesis of PTH, as prolonged hypercalcemia has been shown
to depress the levels of preproPTH mRNA in bovine and human
adenomatous parathyroid tissue. Prolonged hypercalcemia
also depresses parathyroid cell proliferation in vitro, so
calcimimetics can also be effective in limiting the
parathyroid cell hyperplasia characteristic of secondary HPT.
        Other cells in the body can respond directly to
physiological changes in the concentration of extracellular
Ca^{2+}. Calcitonin secretion from parafollicular cells in
the thyroid (C-cells) is regulated by changes in the
concentration of extracellular Ca^{2+}. Renin secretion from
juxtaglomerular cells in the kidney, like PTH secretion, is
depressed by increased concentrations of extracellular
Ca^{2+}. Extracellular Ca^{2+} causes the mobilization of
intracellular Ca^{2+} in these cells. Other kidney cells
respond to calcium as follows: elevated Ca^{2+} inhibits
formation of $1,25(OH)_2$ Vitamin D by proximal tubular
cells, stimulates production of calcium-binding protein in
distal tubular cells, and inhibits tubular reabsorption of
Ca^{2+} and Mg^{2+} and the action of vasopressin on the
medullary thick ascending limb of Henle's loop (MTAL),
reduces vasopressin action in the cortical collecting duct
cells, and affects vascular smooth muscle cells in blood
vessels of the renal glomerulus. Calcium promotes the
differentiation of intestinal goblet cells, mammary cells,
and skin cells. It also inhibits atrial natriuretic peptide
secretion from cardiac atria, reduces cAMP accumulation in
platelets, alters gastrin and glucagon secretion, and acts
on vascular smooth muscle cells to modify cell secretion of
vasoactive factors. Isolated osteoclasts respond to

- 123 -

increases in the concentration of extracellular Ca^{2+} with corresponding increases in $[Ca^{2+}]_i$ that arise partly from the mobilization of intracellular Ca^{2+}. Increases in $[Ca^{2+}]_i$ in osteoclasts are associated with an inhibition of functional responses (bone resorption) analogous to PTH secretion in parathyroid cells. Release of alkaline phosphatase from bone-forming osteoblasts is directly stimulated by calcium. Thus, there are sufficient indications to suggest that Ca^{2+}, in addition to its ubiquitous role as an intracellular signal, also functions as an extracellular signal to regulate the responses of certain specialized cells. Molecules of this invention can be used in the treatment of diseases associated with disrupted Ca^{2+} responses in these cells.

     Cloning the Ca^{2+} receptor on parathyroid cells and other cells will allow the presence of homologous proteins in other cells to be directly assessed. A family of structurally homologous Ca^{2+} receptor proteins can thus be obtained. Such receptors will allow understanding of how these cells detect extracellular Ca^{2+} and enable evaluation of the mechanism(s) as a site of action for the therapeutics described herein effective in the treatment of HPT, osteoporosis, and hypertension, and novel therapies for other bone and mineral-related diseases.

     Other uses are discussed above. For example, recombinant Ca^{2+} receptor proteins may be used in therapy, and introduced by standard methods, e.g., by transfection of nucleic acid encoding that protein. In addition, such protein is useful in assays for calcimimetic molecules of this invention.

- 124 -

The following examples illustrate the invention but do not limit its scope.

Examples

In the studies described herein, a variety of organic molecules were found to mobilize intracellular Ca^{2+} and depress PTH secretion in parathyroid cells. These molecules are structurally diverse but most have a net positive charge at physiological pH. The cationic nature of the organic molecules plays an important role but is not the sole factor determining activity.

Example 1: Screening Calcimimetic Molecules on Bovine Parathyroid cells

Dissociated bovine parathyroid cells were purified on gradients of Percoll and cultured overnight in serum- free medium. The cells were subsequently loaded with fura-2 and the concentration of free intracellular Ca^{2+} measured fluorimetricly. Changes in $[Ca^{2+}]_i$ were used to screen for molecules active at the Ca^{2+} receptor. To be considered a calcimimetic, a molecule was required to show the normal effects caused by increasing extracellular Ca^{2+} and triggered by the activation of the Ca^{2+} receptor. That is,

1)   The molecule must elicit an increase in $[Ca^{2+}]_i$; the increase in $[Ca^{2+}]_i$ may persist in the absence of extracellular Ca^{2+} and/or the molecule may potentiate increases in $[Ca^{2+}]_i$ elicited by extracellular Ca^{2+}.

2)   The molecule must cause a decrease in isoproterenol-stimulated cyclic AMP formation which is blocked by pertussis toxin;

- 125 -

3)   The molecule must inhibit PTH secretion over the same range of concentrations that cause the increase in $[Ca^{2+}]_i$; and

4)   The concentration-response curves for increases in $[Ca^{2+}]_i$ and PTH secretion by the molecule must be shifted to the right by a PKC activator, such as phorbol myristate acetate (PMA).

Several structurally different classes of molecules were tested: polyamines, aminoglycoside antibiotics, protamine, and polymers of lysine or arginine. The structures of these molecules are depicted in Figure 1. Included in Figure 1 are the net positive charge of the molecules and their EC_{50}'s for evoking the mobilization of intracellular Ca^{2+} in bovine parathyroid cells.

In general, the greater the net positive charge on the molecule, the greater its potency in causing the mobilization of intracellular Ca^{2+}. However, some striking exceptions to this apparent rule have been found as discussed below.

As can be seen from the figures, spermine, neomycin B, and protamine evoked rapid and transient increases in $[Ca^{2+}]_i$ in fura-2-loaded bovine parathyroid cells (Figs. 6, 7, 11). They did not, however, cause sustained, steady-state increases in $[Ca^{2+}]_i$ in bovine parathyroid cells (Fig. 6, 11), although they did in human parathyroid cells (Fig. 19). In this respect, they resembled the cytosolic Ca^{2+} response elicited by extracellular Mg^{2+}, which causes the mobilization of intracellular Ca^{2+} unaccompanied by an influx of extracellular Ca^{2+} in bovine cells (Fig. 11b). Transient

increases in $[Ca^{2+}]_i$ elicited by spermine, neomycin B,
or protamine were not blocked by low concentrations (1 μM)
of La^{3+} or Gd^{3+} (Fig. 11f,g). Cytosolic Ca^{2+}
transients elicited by the molecular polycations persisted
in the absence of extracellular Ca^{2+} but were blocked when
cellular stores of Ca^{2+} were depleted by pretreatment with
ionomycin (Figs. 7; 11h,i). All these molecules therefore
cause the mobilization of intracellular Ca^{2+} in
parathyroid cells.

It was additionally shown that the molecular
polycations mobilized the same pool of intracellular Ca^{2+}
as that used by extracellular Ca^{2+}. Thus, increasing the
concentration of extracellular Ca^{2+} progressively
inhibited the transient increases in $[Ca^{2+}]_i$ evoked by
spermine (Fig. 6). Conversely, a maximally effective
concentration of spermine or neomycin B (Fig. 12) blocked
transient, but not steady-state increases in $[Ca^{2+}]_i$
evoked by extracellular Ca^{2+}.

Significantly, spermine, neomycin B, and
protamine inhibited PTH secretion to the same extent as
extracellular Ca^{2+}. These inhibitory effects on secretion
were obtained at concentrations that caused the mobilization
of intracellular Ca^{2+} (Figs. 8, 13). These findings are
relevant to understanding the mechanisms contributing to the
regulation of PTH secretion by extracellular Ca^{2+}.
Because a variety of inorganic polycations all inhibit
secretion, yet only extracellular Ca^{2+} causes sustained,
steady-state increases in $[Ca^{2+}]_i$, such increases in
$[Ca^{2+}]_i$ cannot be importantly involved in the regulation
of secretion. Mobilization of intracellular Ca^{2+}, rather

than the influx of extracellular Ca^{2+}, is the essential
mechanism associated with the inhibition of PTH secretion.
This is important because it defines the sufficient
mechanism to be affected if a molecule is to affect PTH
secretion; molecules stimulating selectively the influx of
extracellular Ca^{2+} will be relatively ineffective in
suppressing PTH secretion. In contrast, molecules causing
solely the mobilization of intracellular Ca^{2+} should be
just as efficacious as extracellular Ca^{2+} in suppressing
PTH secretion.

Like the mobilization of intracellular Ca^{2+}
elicited by extracellular Ca^{2+}, that elicited by molecular
polycations was depressed by PMA. A representative
experiment showing the preferential inhibitory effects of
PMA on cytosolic Ca^{2+} transients elicited by spermine is
shown in Fig. 14. Cytosolic Ca^{2+} transients evoked by ATP
were unaffected, even when a submaximal concentration of ATP
was used. The effect of PMA on cytosolic Ca^{2+} transients
elicited by the molecular polycations paralleled its effect
on responses to extracellular Ca^{2+}; in both cases there
was a shift to the right in the concentration-response curve
(Fig. 15). The depressive effects of PMA on $[Ca^{2+}]_i$
were accompanied by potentiating effects on secretion which
were overcome at higher concentrations of the organic
polycations (Fig. 16).

The mobilization of intracellular Ca^{2+}
elicited by molecular polycations was associated with
increases in the formation of inositol phosphates. For
example, protamine caused a rapid (within 30 s) increase in
the formation of IP_3 which was accompanied by a rise in

levels of IP_1. Both these effects were dependent on the
concentration of extracellular protamine (Fig. 17).
Moreover, pretreatment with PMA blunted the formation of
inositol phosphates elicited by molecular polycations.
Representative results obtained with spermine are presented
in Fig. 18.

Spermine, neomycin B, and protamine depressed
isoproterenol-induced increases in cyclic AMP. Like the
inhibitory effects of extracellular Ca^{2+} on cyclic AMP
formation, those caused by molecular polycations were
blocked by pretreatment with pertussis toxin (Table 2).

Table 2

	cyclic AMP (% of control)	
	control	+PTx
0.5 mM Ca^{2+}	100	106 $\pm$ 8
2.0 mM Ca^{2+}	19 $\pm$ 4	94 $\pm$ 2
0.5 mM Ca^{2+}, 200 μM Spermine	23 $\pm$ 5	93 $\pm$ 6
0.5 mM Ca^{2+}, 30 μM Neomycin B	28 $\pm$ 8	87 $\pm$ 6
0.5 mM Ca^{2+}, 2 μg/ml Protamine	20 $\pm$ 4	89 $\pm$ 9

Pertussis toxin (PTx) blocks the inhibitory effects of
extracellular Ca^{2+} and molecular polycations on cyclic AMP
formation. Bovine parathyroid cells were cultured for 16 h
with or without 100 ng/ml pertussis toxin. The cells were
subsequently washed and incubated for 15 min with 10 μM
isoproterenol with or without the indicated concentrations
of extracellular Ca^{2+} or molecular polycations. Total
cyclic AMP (cells + supernatant) was determined by RIA and
the results are expressed as a percentage of the levels

obtained in 0.5 mM Ca^{2+} (112 $\pm$ 17 pmole/10^6 cells).
Each value is the mean $\pm$ SEM of three experiments.

In human parathyroid cells, extracellular Mg^{2+}
elicited a sustained, steady-state increase in $[Ca^{2+}]_i$
in addition to a rapid transient increase (Fig. 10). As in
bovine parathyroid cells responding to extracellular Ca^{2+},
the steady-state increase in $[Ca^{2+}]_i$ evoked by Mg^{2+} in
human parathyroid cells results from Ca^{2+} influx through
voltage-insensitive channels (Fig. 10a). This effect of
Mg^{2+} on steady-state $[Ca^{2+}]_i$ in human parathyroid
cells is seen in both adenomatous and hyperplastic tissue.

Neomycin B and spermine were tested for effects
on $[Ca^{2+}]_i$ in human parathyroid cells prepared from
adenomatous tissue. Representative results with neomycin B
are shown in Fig. 19. Neomycin B caused not only a
transient but additionally a steady-state increase in
$[Ca^{2+}]_i$ in human parathyroid cells (Fig. 19a). Thus, in
human cells, the pattern of change in $[Ca^{2+}]_i$ evoked by
extracellular Ca^{2+}, Mg^{2+} or neomycin B is very similar.

Cytosolic Ca^{2+} transients elicited by neomycin
B persisted in the presence of La^{3+} (1μM) and absence of
extracellular Ca^{2+}. Neomycin B therefore causes the
mobilization of intracellular Ca^{2+} in human parathyroid
cells. Neomycin B inhibited PTH secretion from human
parathyroid cells at concentrations that caused the
mobilization of intracellular Ca^{2+} (Fig. 13). There were,
however, some differences in the responses of human and
bovine parathyroid cells to neomycin B. The EC_{50} of
neomycin B for the mobilization of intracellular Ca^{2+} was
40 μM in bovine and 20 μM in human parathyroid cells

(cf. Figs. 13 and 15), whereas the potency of spermine was similar in bovine and human parathyroid cells (EC_{50} = 150 µM). Thus, although bovine cells can be used for initial studies to screen test molecules for activity, it is important to perform follow-up studies using human parathyroid cells.

To assess the effects of molecular polycations on C-cells, a neoplastic cell line, derived from a rat medullary thyroid carcinoma (rMTC 6-23 cells) was used. Both spermine (10 mM) and neomycin B (5 mM) were without effect on basal $[Ca^{2+}]_i$ in these cells. Nor did either molecule affect the response to the subsequent addition of extracellular Ca^{2+}. Representative results documenting the lack of effect of neomycin B are shown in Fig 21. Neomycin B (1 mM) or spermine (1 or 5 mM) failed to evoke any increase in $[Ca^{2+}]_i$ in osteoclasts (Fig. 23). In the trace shown, there appeared to be some potentiation of the response to a subsequent increase in the concentration of extracellular Ca^{2+}, although this was not a consistent finding. In two other cells, spermine (5 mM) was again without effect on basal $[Ca^{2+}]_i$ and caused a small inhibition (about 15%) of the extracellular Ca^{2+}-induced increase in $[Ca^{2+}]_i$. In a third cell, neomycin B (5 mM) was without effect on basal $[Ca^{2+}]_i$ and did not affect increases in $[Ca^{2+}]_i$ elicited by extracellular Ca^{2+}. The overall picture that develops from these studies is that spermine and neo mycin B are without effect on basal or stimulated levels of cytosolic Ca^{2+} in osteoclasts.

The failure of the molecular polycations to affect the Ca^{2+}-sensing mechanisms of C-cells or

- 131 -

osteoclasts demonstrates the ability to discover or design
novel lead molecules that act specifically on the
parathyroid cell Ca^{2+} receptor or otherwise modulate one
or more functions of the parathyroid cell's normal response
to $[Ca^{2+}]$.

Screening of various other molecules is
described in detail below and the results summarized in
Table 1.

Example 2:   Polyamine Screening

Straight-chain polyamines (spermine, spermidine,
TETA, TEPA, and PEHA) and two derivatives thereof (NPS 381
and NPS 382) were screened as in Example 1. These molecules
were all found to mobilize intracellular Ca^{2+} in bovine
parathyroid cells. Their order of potency is as follows,
with the net positive charge listed in parentheses:

Table 3

Molecule	EC_{50} (in μM)
NPS 382 (+8)	50
NPS 381 (+10)	100
spermine (+4)	150
PEHA (+6)	500
spermidine (+3)	2000
TEPA (+5)	2500
TETA (+4)	8000

Putrescine (+2) and cadaverine (+2) were
inactive at a concentration of 2mM.

Another straight-chain polyamine, DADD, behaved
somewhat differently from the other polyamines and is

- 132 -

described in Example 7.

Example 3: Cyclic Polyamine Screening

Two cyclic polyamines, hexacyclen and NPS 383, were screened as in Example 1N Hexacyclen (+6, EC_{50} = 20 µM) is 7-fold more potent than NPS 383 (+8, EC_{50} = 150 µM). The converse would be expected based solely on net positive charge as the structural characteristic for Ca^{2+} receptor activity.

Example 4: Aminoglycoside Antibiotic Screening

Six antibiotics were screened as in Example 1. The resulting EC_{50}'s for the mobilization of intracellular Ca^{2+}, in rank order of potency, were:

Table 4

Antibiotic	EC_{50} (in µM)
neomycin (+6)	10
gentamicin (+5)	150
bekanamycin (+5)	200
streptomycin (+3)	600

Kanamycin (+4.5) and lincomycin (+1) were without effect at a concentration of 500 µM. Within the aminoglycoside series, there is a correlation between net positive charge and potency. However, neomycin is considerably more potent than various polyamines (NPS 381, NPS 382, NPS 383, PEHA) that have an equal or greater positive charge. Since aminoglycoside antibiotics of this type have renal toxicity which may be related to interaction with calcium receptors in the kidney, such screening could be used to screen for toxicity in the development of new aminoglycoside antibiotics.

Example 5: Peptide and Polyamino Acid Screening

Protamine and polymers of lysine or arginine varying in peptide length were screened for their ability to mobilize intracellular Ca^{2+} as in Example 1. The resulting EC_{50}'s for the mobilization of intracellular Ca^{2+}, in rank order of potency, were:

Table 5

Peptide (MW in kD)	EC_{50}(in nM)
polyArg (100)	4
polyArg (40)	15
polyLys (27)	30
protamine (4.8)	75
polyArgTyr (22)	200
polyLys (14)	1000
polyLys (3.8)	3000

The net positive charge of these polymers increases as the MW increases. Thus, as for the aminoglycosides, there is a direct correlation between net charge and potency among this series of polyamino acids. Protamine is essentially polyArg with a net positive charge of +21.

Example 6: Arylalkylamine Screening

Molecules selected from the class of arylalkylamine toxins derived from the venoms of wasps and spiders were screened as in Example 1.

Philanthotoxin-433 (+3) was without effect at a concentration of 500 μM. It is similar in structure to the argiotoxins described below.

- 134 -

Argiotoxin-636 (400 μM) did not elicit increases in $[Ca^{2+}]_i$ but it did potentiate cytosolic Ca^{2+} responses to the subsequent addition of extracellular Ca^{2+}. This is a feature common to all molecules that activate the Ca^{2+} receptor and is also seen with a variety of extracellular divalent cations. This is considered in more detail in Example 7.

In contrast to argiotoxin-636, argiotoxin-659 elicited increases in $[Ca^{2+}]_i$ with an EC_{50} of 300 μM. Argiotoxin-659 differs from argiotoxin-636 in having a hydroxylated indole moiety rather than a dihydroxyphenyl group. This is the only difference in the structure of these two molecules. Thus, the difference in potency lies in the nature of the aromatic group, not in the polyamine chain which carries the positive charge.

Example 7: Screening of Ca^{2+} Channel Blockers

Ca^{2+} channel blockers, i.e., those molecules which block influx of extracellular Ca^{2+} through voltage- sensitive Ca^{2+} channels, were screened as in Example 1. There are three structural classes of Ca^{2+} channel blockers: (1) dihydropyridines, (2) phenylalkylamines, and (3) benzothiazipines.

None of the dihydropyridines tested (nifedipine, nitrendipine, BAY K 8644, and (-) 202-791 and (+) 202-791) had any effect on basal $[Ca^{2+}]_i$ or increases in $[Ca^{2+}]_i$ evoked by extracellular Ca^{2+} when they were tested at 1 μM. Previous studies showed that parathyroid cells lack voltage-sensitive Ca^{2+} channels, but do have voltage-insensitive Ca^{2+} channels that are regulated by the Ca^{2+} receptor.

- 135 -

The phenylalkylamines examined were verapamil, D-600 (a methoxy-derivative of verapamil), TMB-8, and an analog of TMB-8, NPS 384. The first three molecules were tested at a concentration of 100 μM. The phenylalkylamines behaved differently from other molecules examined. They evoked no change in $[Ca^{2+}]_i$ when added to cells bathed in buffer containing a low concentration of extracellular Ca^{2+} (0.5 mM). However, verapamil, D-600, and TMB-8 potentiated the mobilization of intracellular Ca^{2+} elicited by extracellular divalent cations and they additionally blocked the influx of extracellular Ca^{2+}. At intermediate levels of extracellular Ca^{2+} (1-1.5 mM), these molecules were capable of evoking a small but robust increase in $[Ca^{2+}]_i$ that arose from the mobilization of intracellular Ca^{2+}.

The phenylalkylamines act differently than organic polycations like neomycin. The data suggest that verapamil, D-600 and TMB-8 are partial agonists or allosteric activators at the Ca^{2+} receptor, in contrast to the other molecules examined which are full agonists.

Molecule NPS 384, at a concentration of 300 μM, did not evoke an increase in $[Ca^{2+}]_i$ but it blocked influx of extracellular Ca^{2+}. Testing at higher concentrations may reveal an ability of this molecule to cause the mobilization of intracellular Ca^{2+}.

While the ability of these molecules to block influx is intriguing and not entirely unexpected, it is the ability of these molecules to evoke transient

increases in $[Ca^{2+}]_i$ (arising from intracellular Ca^{2+} mobilization) that is important. Considerable experience with measurements of $[Ca^{2+}]_i$ in parathyroid cells shows that transient increases in $[Ca^{2+}]_i$ almost invariably result from the mobilization of intracellular Ca^{2+} and therefore reflects activation of the Ca^{2+} receptor.

The benzothiazipine examined, diltiazem, was similar in all respects to verapamil and D-600 and was also effective at 100 µM.

It should be mentioned that with the exception of the phenylalkylamines, all the active molecules tested above evoke increase in $[Ca^{2+}]_i$ that are of magnitude similar to that evoked by a maximally effective concentration of extracellular Ca^{2+}. This shows that these molecules are equally efficacious as extracellular divalent cations. This contrasts with the activity of phenylalkylamines, which seem to act only as partial agonists.

Amongst the phenylalkylamines, some interesting structure-activity relationships emerge. Significant is the different potencies of molecules like TMB-8 and NPS 384. TMB-8 potentiated transient increases in $[Ca^{2+}]_i$ at 100 µM whereas NPS 384 fails to do so even at 300 µM, yet these molecules carry the same net positive charge. It follows that some other structural feature, unrelated to net charge, imparts greater potency to TMB-8.

Example 8:   Molecule Screening on Human Parathyroid Cells

Spermine and neomycin were tested for effects on $[Ca^{2+}]_i$ in human parathyroid cells obtained from glands removed by surgery and prepared as in Example 1. In human parathyroid cells, spermine was found to cause only a small increase in $[Ca^{2+}]_i$ when tested at a concentration of 300 µM.

Neomycin, on the other hand, evoked a large increase in $[Ca^{2+}]_i$ in human parathyroid cells when tested at a concentration of 20 µM. The magnitude of the response elicited by neomycin was equal to that evoked by a maximally effective concentration of extracellular Ca^{2+}.

Example 9:   Molecule Screening on Xenopus Oocytes

Oocytes injected with mRNA from human parathyroid cells express the Ca^{2+} receptor and mobilize intracellular Ca^{2+} in response to a variety of extracellular inorganic di- and trivalent cations. Using this screen allows one to test for an action directly on the Ca^{2+} receptor. Oocytes expressing the Ca^{2+} receptor also responded to several molecules active on intact parathyroid cells when screened as follows. Hexacyclen caused the mobilization of intracellular Ca^{2+} at a concentration of 135 µM. Neomycin (100 µM) and NPS 382 (5 mM) were also effective. This offers rather compelling evidence showing that these molecules act on the Ca^{2+} receptor or on some other protein intimately associated with its function.

For example, we have been able to detect Ca^{2+}

receptor expression in oocytes by measuring $^{45}Ca^{2+}$
mobilization. In these experiments, oocytes were
injected with bovine parathyroid mRNA or water and,
after 72 hours exposed to serum or 10 mM neomycin.
Prior to being stimulated, oocytes were loaded with
$^{45}Ca^{2+}$. Stimulation with serum for 20 min resulted
in intracellular $^{45}Ca^{2+}$ release representing a 45%
increase compared to mock challenge with buffer.
Challenge with 10 mM neomycin for 20 min. resulted in a
76% increase in $^{45}Ca^{2+}$ release. The assay is
sensitive enough for use in cloning the Ca^{2+} receptor,
and has the advantage of a higher throughout than the
electrophysiological measurement of Ca^{2+} activated
Cl^- current.

     In another example, human osteoclastoma tissue
was obtained from bone biopsy tissue. Oocytes injected
with mRNA isolated from this tissue were challenged with
30 mM Ca^{2+}. Controls did not respond while 8 of 12
oocytes injected with osteoclastoma mRNA responded
appropriately (Fig. 34). These experiments provide the
first evidence that the Ca^{2+} response of osteoclasts
to extracellular Ca^{2+} is in fact genetically encoded.
The results also indicate that the osteoclast Ca^{2+}
receptor may be cloned by expression in Xenopus oocytes.

Example 10: Molecule Screening on Rat Osteoclasts

     The different sensitivities of parathyroid cells
and rat osteoclasts to extracellular Ca^{2+} suggest that
their Ca^{2+} receptors are different. While parathyroid
cells respond to extracellular Ca^{2+} concentrations
between 0.5 and 3 mM, osteoclasts respond only when the

- 139 -

level of extracellular Ca^{2+} increases beyond 5 mM.
This rather high concentration of Ca^{2+} is nonetheless
physiological for osteoclasts; as they resorb bone, the
local concentration of extracellular Ca^{2+} may reach
levels as high as 30 mM.

Molecule screening with rat osteoclasts was
performed as follows. Osteoclasts were obtained from
the long bones of neonatal rats. $[Ca^{2+}]_i$ was
measured in single cells using the fluorimetric
indicator indo-1. Spermine, spermidine, neomycin, and
verapamil were tested, and none of these caused any
large increase in $[Ca^{2+}]_i$ in osteoclasts (although
small responses were detected).

At a concentration of 1 mM, spermidine caused a
small increase in $[Ca^{2+}]_i$ (about 10% of that evoked
by a maximal concentration of extracellular Ca^{2+}).
Neither neomycin (10 mM) nor spermine (10 or 20 mM)
caused increases in $[Ca^{2+}]_i$ in rat osteoclasts.
Neomycin (10 mM) did not block the increase in
$[Ca^{2+}]_i$ elicited by the subsequent addition of 25 mM
extracellular Ca^{2+}. Pretreatment with spermine (20
mM), however, did depress the response to extracellular
Ca^{2+}. Verapamil (100 μM) caused no detectable
increase in $[Ca^{2+}]_i$ but it did block the response to
extracellular Ca^{2+}.

Comparisons between osteoclasts and parathyroid
cells show that molecules active on the latter are
relatively ineffective in osteoclasts. This
demonstrates that drugs that target a specific Ca^{2+}
receptor without affecting those receptor types present

- 140 -

on other Ca^{2+}-sensing cells are readily developed.
Similarly, drugs active at two or more such Ca^{2+}
receptors may also be developed.

Screening for Calcimimetic and Calcilytic Activity on
the Osteoclast Calcium Receptor

Compounds possessing activity on the osteoclast
calcium receptor can be discovered by measuring
$[Ca^{2+}]_i$ in single rat osteoclasts as described
above. An improved assay enables moderate-to-high
levels of compound throughout. This new method is
based on the use of rabbit osteoclasts which can be
obtained in high yield (10^5 per animal) and purity
(95% of the cells are osteoclasts). The purity of the
rabbit osteoclast preparation allows measurements of
$[Ca^{2+}]_i$ to be performed on populations of cells.
Because the recorded fluorescence signal is an averaged
population response, intercellular variability is
minimized and the precision of the assay is greatly
increased. This, in turn, enables more compounds to be
screened for activity.

Rabbit osteoclasts are prepared from 6-day old
bunnies. The animals are sacrificed by decapitation and
the long bones removed and placed into osteoclast medium
(OC medium: alpha-minimum essential medium containing 5%
fetal bovine serum and penicillin/streptomycin). The
bones are cut into sections with a scalpel and placed in
2 ml of OC media in a 50 ml conical centrifuge tube.
The bone sections are minced with scissors until a
fairly homogeneous suspension of bone particles is
obtained. The suspension is then diluted with 25 ml of

OC media and the preparation swirled gently ("vortexed")
for 30 seconds. The bone particles are allowed to
settle for 2 minutes after which the supernatant is
removed and added to a 50 ml centrifuge tube. The bone
particles are resuspended in OC media, swirled,
sedimented and harvested as just described. The
supernatants from the two harvests are combined and
centrifuged and the resulting cellular pellet
resuspended in Percoll. The suspension is then
centrifuged and the white viscous band just below the
meniscus is removed and washed with OC media. The
Percoll centrifugation step results in a significant
improvement in purity and allows osteoclasts to be
plated at high densities, suitable for measuring
$[Ca^{2+}]_i$ in populations of cells. The cells are
plated onto glass cover slips appropriate for measuring
$[Ca^{2+}]_i$ according to one of the methods described
below. If necessary, the purity of the preparation can
be improved. In this case, the cells are cultured
overnight and then rinsed with $Ca^{2+}-$ and $Mg^{2+}-$free
buffer. The cell monolayer is then immersed in $Ca^{2+}-$
and $Mg^{2+}-$free buffer containing 0.02% EDTA and 0.001%
pronase for 5 min. This buffer is then removed and
replaced with OC media and the cells allowed to recover
for 1 to 2 hours before loading the cells with
fluorimetric indicator and measuring $[Ca^{2+}]_i$ as
described below.

    In one embodiment, this technique allows the
measurement of $[Ca^{2+}]_i$ in populations of osteoclasts
using fluorescence microscopy. The purified osteoclasts

are allowed to attach to 25 mm diameter glass cover
slips and then loaded with indo-1. The cover slips are
secured into a superfusion chamber and placed onto the
stage of a fluorescence microscope. The use of a low
power objective (x4) allows a field containing 10 to 15
osteoclasts to be visualized. In one variation, the
fluorescence of each cell in the field can be recorded
simultaneously and stored separately for later
analysis. Changes in $[Ca^{2+}]_i$ of each cell can be
estimated and the average response of all cells in the
field calculated.   In another variation, the
fluorescence from the entire field of cells can be
recorded and processed immediately. In either
variation, the final data is in the form of an average
response from the cells present in the microscopic
field. Because of this, intercellular variability is
minimized and precision of the assay greatly increased.
This method enables 10-20 compounds per week to be
screened for activity on the osteoclast calcium receptor.
          In a more preferred embodiment, this technique
allows the measurement of $[Ca^{2+}]_i$ in populations of
osteoclasts using a conventional fluorimeter. The
purified osteoclasts are allowed to attach to
rectangular glass cover slips. In one variation, a
standard quartz cuvette ($1cm^2$) is used and the glass
coverslips are 2 x 1.35 cm. In another variation, a
microcuvette is used ($0.5\ cm^2$) and the glass
coverslips are 1 x 0.75 cm. In either case the cells
are loaded with fura-2 or some other suitable
fluorimetric indicator for measuring $[Ca^{2+}]_i$. The

- 143 -

fluorescence of indicator-loaded cells is recorded as
described above for bovine parathyroid cells. This
method allows a higher throughput than fluorescence
microscopy and enables 20-50 compounds per week to be
evaluated for activity on the osteoclast calcium
receptor.

In a most preferred embodiment, the technique can
be used to measure $[Ca^{2+}]_i$ in osteoclasts in a
96-well plate. The purified osteoclasts are plated at
high density into each well of a 96-well plate and
subsequently loaded with a suitable fluorimetric
indicator. The fluorescence of each well is recorded
using a fluorimetric plate reader. This method has the
potential of becoming fully automated using robotics a
would enable high throughput screening in which 50 to
100 compounds per week could be screened.

Other Ca^{2+} Receptor Examples

The following examples demonstrate that, just as
there are subtypes of receptors for molecular ligands,
so too do there appear to be subtypes of Ca^{2+}
receptors that can be differentially affected by drugs.
The parathyroid cell Ca^{2+} receptor senses levels of
extracellular Ca^{2+} around 1.5 mM whereas the Ca^{2+}
receptor on the osteoclast responds to levels around 10
mM (Fig. 22). Neomycin or spermine, which activate the
parathyroid cell Ca^{2+} receptor, fail to affect the
Ca^{2+} receptors on C-cells or osteoclasts (Figs. 21 and
23). These data constitute the first evidence for
pharmacologically distinct subtypes of Ca^{2+} receptors
and these data are being used to design and develop

- 144 -

drugs that act selectively on a particular type of Ca^{2+} receptor. Indeed, testing of lead molecules demonstrate such cell-specific effects. For example, NPS 449, which elicits increases in $[Ca^{2+}]_i$ in osteoclasts is without effect on $[Ca^{2+}]_i$ in parathyroid cells. Conversely, NPS 447, which activates the parathyroid cell Ca^{2+} receptor, is effective in activating the osteoclast Ca^{2+} receptor only at concentrations 10-fold higher. Finally, agatoxin 489, although not very potent in activating the C-cell Ca^{2+} receptor (EC_{50} = 150 μM), is a quite potent activator of the parathyroid cell Ca^{2+} receptor (EC_{50} = 3 μM). The lead molecules presently under development will affect selectively the activity of a specific type of Ca^{2+}-sensing cell in vivo.

Drugs with less specificity might not necessarily be therapeutically undesirable. Thus, depressing osteoclast activity and stimulating calcitonin secretion are two different approaches to inhibiting bone resorption. Drugs that target the Ca^{2+} receptors on both of these cells might be very effective therapies for osteoporosis. Because PTH is also involved in regulating bone metabolism, drugs acting on the parathyroid cell Ca^{2+} receptor may also be useful in the treatment and/or prevention of osteoporosis.

Results of some test molecules are shown below. In Table 6, the comparative activity of calcimimetic molecules is shown. Bovine parathyroid cells and C-cells (rMTC 6-23 cells) were loaded with fura-2, and rat osteoclasts with indo-1 and the potency of the indicated

- 145 -

molecules to mobilize intracellular Ca^{2+} determined by constructing cumulative concentration-response curves. Molecules listed as "inactive" did not alter $[Ca^{2+}]_i$ when tested at a concentration of 1 mM.

Table 6

| | $EC_{50}(\mu M)$ | | |
COMPOUND	PARATHYROID	OSTEOCLAST	C-CELL
NPS R-568	0.60	200	>300
NPS S-568	30	---	---
NPS R-467	2	>100	---
NPS S-467	>30	---	---
NPS 017	6	inactive	150
NPS 447	9	150	---
NPS 456*	15	200	>100
NPS 015	22	---	inactive
NPS 109	40	>300	5
NPS 449	inactive	150	---
NPS 468*	30	250	---
spermine	150	inactive	inactive
neomycin	40	inactive	inactive

*racemic mixture; *inactive* is defined as causing no increase in cytosolic Ca⁺ at a concentration of 1-5 mM.

*racemic mixture; "inactive" is defined as causing no increase in cytosolic Ca^{2+} at a concentration of 1-5 mM.

Example 11: Lead Molecules for Parathyroid Ca^{2+} Receptor

Structure-activity studies using polyamines and arylalkylamines led to the testing of molecules

- 146 -

structurally akin to NPS 456. NPS 456 is a potent
activator of the parathyroid cell Ca^{2+} receptor. This
molecule is notable because it possess only one positive
charge yet is much more potent than many polybasic
molecules. Brief (2 min) pretreatment with PMA shifts
the concentration-response curve for NPS 456 to the
right. This indicates that NPS 456 acts through the
same mechanism used by extracellular Ca^{2+}. NPS 456
evokes the mobilization of intracellular Ca^{2+} in
<u>Xenopus</u> oocytes expressing the parathyroid cell Ca^{2+}
receptor, which demonstrates a direct action on the
Ca^{2+} receptor (Fig. 33). Moreover, NPS 456 contains a
chiral carbon, and therefore exists in two isomeric
forms. Both isomers have been synthesized and examined
for activity. The R-isomer, NPS 447, is 12 times more
potent than the S-isomer, NPS 448 (Fig. 28). This is
the first demonstration that a Ca^{2+} receptor can
recognize an organic molecule in a stereospecific
manner.

Because NPS 447 is a structurally simple molecule
with selective and potent effects on the parathyroid
cell Ca^{2+} receptor, structure-activity studies around
this lead molecule are simple. The aim of these studies
is to generate an array of related molecules with
various characteristics from which the final development
candidate can be selected. This effort has already
revealed some of the structural domains of NPS 447 that
contribute to activity and potency. For example, the
novel compound NPS 459 is an analog of NPS 447 that is
smaller (MW < 240) yet nearly as potent as the parent

molecule, whereas several other analogs are relatively
inactive. The most interesting molecules from this
analog project can be put into in vivo testing for
effects on PTH secretion and serum Ca^{2+} levels (see
Examples 15, 16, 17, 18 and 23).

The novel compound NPS 467 is an even smaller
molecule than NPS 447 yet the former is about 3-fold
more potent than the latter in causing increases in
$[Ca^{2+}]_i$ in parathyroid cells. Like NPS 456, NPS 467
is a racemic mixture. Resolution of NPS 467 into its
enantiomers provides an isomer of even greater potency
than the racemic mixture (see Example 16). Further
structure-activity studies on molecules related to NPS
447, NPS 467 and NPS 568 are expected to yield pure
isomers with greater potency than these molecules in
their racemic forms.

Results obtained with NPS 456 (Fig. 33) show that
it elicits oscillatory increases in Cl^- current at
concentrations of 100 µM. NPS 456 is the most potent
molecule activate on Xenopus oocytes expressing the
parathyroid cell Ca^{2+} receptor. The results obtained
in this expression system with neomycin and NPS 456
demonstrate that these molecules act directly on the
Ca^{2+} receptor.

Example 12: Osteoclast Ca^{2+} Receptor Lead Molecules

The strategy used for elucidating the mechanism
of action of extracellular Ca^{2+} on the osteoclast was
similar to that proven effective in parathyroid cells.
The first experiments examined the effects of La^{3+} on
$[Ca^{2+}]_i$ in single rat osteoclasts loaded with the

fluorimetric indicator indo-1. As described above, trivalent cations like La^{3+} are impermeant and block Ca^{2+} influx. Low micromolar concentrations of La^{3+} partially depressed extracellular Ca^{2+}-induced increases in $[Ca^{2+}]_i$ (Fig. 29). The demonstration of a La^{3+}-resistant increase in $[Ca^{2+}]_i$ provides evidence for the mobilization of intracellular Ca^{2+}. The results of these experiments parallel those obtained in parathyroid cells and suggest that similar mechanisms are used by extracellular Ca^{2+} to regulate $[Ca^{2+}]_i$ in both cell types.

Another series of experiments showed that extracellular Mn^{2+} evoked transient increases in $[Ca^{2+}]_i$ (Fig. 30(a)) that persisted in the absence of extracellular Ca^{2+} (Fig. 30B). These results are likewise indicative of the mobilization of intracellular Ca^{2+}. Although Mn^{2+} can enter some cells, it is unlikely to do so in the osteoclast because Mn^{2+} quenches the fluorescence of indo-1. Thus, if Mn^{2+} penetrated intracellularly, a decrease, not an increase in the fluorescent signal would be observed.

The results obtained with a variety of di- and trivalent cations are all consistent with the presence of a Ca^{2+} receptor on the surface of the osteoclast that is coupled to the mobilization of intracellular Ca^{2+} and influx of extracellular Ca^{2+} through voltage-insensitive channels. Results show evidence for genetic material in human osteoclasts that encodes a Ca^{2+} receptor protein (see below). Transient increases in $[Ca^{2+}]_i$ resulting from the mobilization

of intracellular Ca^{2+}, are sufficient to inhibit
osteoclastic bone resorption in vitro. Thus, as with
the parathyroid cell, activation of the Ca^{2+} receptor
appears to be a viable means of inhibiting the activity
of osteoclasts.

NPS 449 is presently the lead molecule for
calcimimetic drugs on this receptor. It is a small
molecule (MW < 425) and it mobilizes intracellular
Ca^{2+} in rat osteoclasts with a EC_{50} of about
150 μM (Figs. 31A and 31B). Although the potency of
NPS 449 is relatively low, it has a simple structure
with only one positive charge and is expected to have
desirable pharmacodynamic and pharmacokinetic
properties.

NPS 449 was examined for its ability to inhibit
bone resorption in vitro. This was done by morphometric
analysis of pit formation on thin slices of bovine
cortical bone using scanning electron microscopy. Rat
osteoclasts were incubated for 24 hours in slices of
bone in the presence or absence of various
concentrations of NPS 449. NPS 449 caused a
concentration-dependent inhibition of bone resorption
with an IC_{50} of 10 μM. The anticipated results
provide the first demonstration that molecules acting at
this novel site can inhibit osteoclastic bone
resorption. More potent analogs of NPS 449 will be
generated using synthetic chemistry and will be tested
and assayed using the methods described herein.

Example 13: C-Cell Ca^{2+} Receptor Lead Molecules

Activation of the C-cell Ca^{2+} receptor
stimulates the secretion of calcitonin which then acts
on osteoclasts to inhibit bone resorption. Calcimimetic
drugs selectively affecting C-cells are useful in the
treatment of osteoporosis.

The mobilization of intracellular Ca^{2+} is used
as a functional index of Ca^{2+} receptor activity. The
screening effort in C-cells is facilitated by the
availability of cultured cell lines expressing the C-
cell phenotype (e.g., rat medullary thyroid carcinoma
cells; rMTC 6-23 cells). Selected for initial study
were three arylalkylamine molecules. Two are naturally
occurring (agatoxin 489 and agatoxin 505) and the other
(NPS 019) is a synthetic agatoxin analog. Agatoxin 505
was found to block extracellular Ca^{2+}-induced
increases in [Ca^{2+}]$_i$, with an IC$_{50}$ of 3 µM. The
inhibitory effect resulted from a block of the L-type
voltage-sensitive Ca^{2+} channel present in these
cells. In contrast, agatoxin 489 was found to mobilize
intracellular Ca^{2+} in rMTC cells with an EC$_{50}$ of
150 µM. This was the first organic molecule
discovered that was found to activate the C-cell Ca^{2+}
receptor. The synthetic analog, NPS 019, was even more
potent and mobilized intracellular Ca^{2+} with an EC$_{50}$
of 5 µM (Fig. 32). It is significant that the only
structural difference between NPS 019 and agatoxin 489
is the presence or absence of an hydroxyl group. The
fact that such subtle differences in structure affect
profoundly the potency of molecules indicates a

- 151 -

structurally specific binding site on the Ca^{2+}
receptor. This, in turn, encourages the view that very
potent and selective activators of Ca^{2+} receptors can
be developed.

NPS 019, which is a small molecule (MW < 500), is
a lead molecule for development of calcimimetics of the
C-cell Ca^{2+} receptor and can be tested for its ability
to stimulate calcitonin secretion in vitro. Subsequent
in vivo testing will then determine the ability of this
molecule to stimulate calcitonin secretion and inhibit
bone resorption. These in vivo studies will be
performed in rats. The results obtained in these
studies, which are anticipated to be positive, will
provide the first evidence showing that a small organic
molecule acting on a novel receptor can stimulate
calcitonin secretion and depress bone resorption.

Example 14: Calcilytic Activity of NPS 021 on
Parathyroid Cells

For a compound to be considered a calcilytic, it
must block the effects of extracellular Ca^{2+} or a
calcimimetic compound on an extracellular Ca^{2+}-sensing
cell. An example of a calcilytic compound is NPS 021,
the structure of which is provided in Fig. 1. In bovine
parathyroid cells loaded with fura-2, NPS 021 blocks
increases in $[Ca^{2+}]_i$ elicited by extracellular
Ca^{2+}. The IC_{50} of NPS 021 for blocking this
response is about 200 àM and, at concentrations around
500 àM, the increase in $[Ca^{2+}]_i$ evoked by
extracellular Ca^{2+} is abolished. Significantly, NPS
021 does not by itself cause any change in $[Ca^{2+}]_i$

when tested at low $[Ca^{2+}]$ (0.5 mM; Fig. 37). Ga^{3+}
is also calcilytic to Xenopus oocytes expressing the
cloned Ca^{2+} receptor: Ga^{3+} by itself has no effect
on the Cl^- currents activated by Gd^{3+}, a
calcimimetic, but pretreatment with Ga^{3+} blocks the
action of Gd^{3+}.

Example 15: NPS 467 Lowers Serum Ionized Calcium

    Compounds shown to activate the bovine
parathyroid cell Ca^{2+} receptor in vitro were tested
for hypocalcemic activity in vivo. Male Sprague-Dawley
rates (200 g) were maintained on a low calcium diet for
one week prior to receiving test substance or vehicle as
control. Blood was collected from the tail vein three
hours after the intra-peritoneal administration of NPS
467. Ionized Ca^{2+} in whole blood or serum was
measured with a Ciba- Corning 634 Analyzer according to
the instructions provided with the instrument. Serum
total calcium, albumin and phosphate were measured by
techniques well known in the art.

    NPS 467 caused a dose-dependent reduction in
serum or whole blood Ca^{2+} (Fig. 38). The fall in
blood Ca^{2+} at this time was paralleled by a
proportional fall in the levels of blood total calcium.
There was no change in serum albumin or phosphate levels
at any of the doses examined. In preliminary studies,
NPS 467, at doses effective in lowering blood Ca^{2+},
caused a dose-dependent reduction in circulating levels
of PTH (Fig. 39). The hypocalcemic effect of NPS 467
was maximal within three hours and returned toward
control levels after 24 hours (Fig. 40).

NPS R-467 (see Example 16) was also effective in lowering serum ionized Ca^{2+} in rats maintained on a normal, calcium-replete diet. A single dose of NPS R-467 (10 mg/kg i.p.) caused a rapid fall in serum levels of ionized Ca^{2+} which were maximal by 1 hr (22% decrease from the control level) and remained depressed at or near this level for up to 6 hours.

Example 16: NPS 467 Lowers Serum Ionized Calcium in a Stereospecific Manner

NPS 467 is a racemic mixture. Resolution of NPS 467 into its two enantiomers was achieved by separation on a chiral column. The R-isomer was about 100-fold more potent than the S-isomer in activating the bovine parathyroid cell Ca^{2+} receptor in vitro as assessed by the ability of the enantiomers to evoke increases in $[Ca^{2+}]_i$ in parathyroid cells (Fig. 41). Likewise, similar resolution of the novel compound NPS 568 into its enantiomers showed that the R-isomer was 40-fold more potent than the S-isomer in causing the mobilization of intracellular Ca^{2+} in bovine parathyroid cells (see Table 6, supra).

The isomers of NPS 467 were examined for effects on serum Ca^{2+} as in Example 15. Consistent with the in vitro results, the R-isomer of NPS 467 proved to be more potent than the S-isomer in lowering serum Ca^{2+} in vivo (Fig. 42; each compound was tested at a concentration of 5 mg/kg body weight).

Example 17: NPS 467 Lowers Serum Ionized Calcium in an in vivo Model of Secondary Hyperparathyroidism

An accepted and widely used animal model of

secondary hyperparathyroidism arising from chronic renal
failure is the 5/6 nephrectomized rat. Animals
receiving such surgery become initially hypocalcemic
and, to maintain serum Ca^{2+} levels, there is a
compensatory hyperplasia of the parathyroid glands and
elevated levels of circulating PTH. Male Sprague-Dawley
rats (250 g) received a 5/6 nephrectomy and were allowed
to recover for 2 weeks. At this time they were
normocalcemic (due to elevated levels of serum PTH).
The administration of NPS R-467 (10 mg/kg i.p.) caused a
rapid (within 2 hours) fall in serum ionized Ca^{2+}
levels to 83% of controls in an animal model of
secondary hyperparathyroidism. This suggests that
compounds of this sort will effectively depress PTH
secretion in patients with secondary hyperparathyroidism
and hyperplastic parathyroid glands.

Example 18: NPS 467 Fails to Lower Serum Ionized
Calcium Levels in Parathyroidectomized Animals

        To determine the primary target tissue upon which
NPS 467 acts to cause a hypocalcemic response, the para
thyroid glands in rats were surgically removed. Animals
receiving a total parathyroidectomy become hypocalcemic
and are largely dependent upon dietary calcium to
maintain serum Ca^{2+} homeostasis. Parathyroidectomized
animals had serum ionized Ca^{2+} levels of 0.92 mM which
fell gradually to 0.76 mM after 6 hours of fasting. The
administration of a single dose of NPS R-467 (10 mg/kg
i.p.) did not cause any change in serum ionized Ca^{2+}
levels over a period of 6 hours. These results
demonstrate that intact parathyroid glands are required

for the hypocalcemic effects of NPS R-467. The data
additionally demonstrate that NPS R-467 can target the
parathyroid glands in vivo. The results are consistent
with the view that NPS R-467 acts on the parathyroid
cell Ca^{2+} receptor in vivo to depress secretion of PTH
and thereby cause serum levels of ionized Ca^{2+} to fall.
Example 19: NPS 467 Increases Intracellular Calcium in
Human Parathyroid Glands

    Dissociated parathyroid cells were prepared from
a parathyroid adenoma obtained by surgery from a patient
with primary hyperparathyroidism. The cells were loaded
with fura-2 and $[Ca^{2+}]_i$ measured as described
above. Both NPS R-467 and NPS R-568 caused
concentration- dependent increase in $[Ca^{2+}]_i$. The
EC_{50}'s for NPS R-467 and NPS R-568 were 20 and 3 μM,
respectively. Both these compounds are thus able to
increase $[Ca^{2+}]_i$ in pathological human tissue and
would thus be expected to decrease serum levels of PTH
and Ca^{2+} in patients with primary hyperparathyroidism.
Example 20: Mechanism of Action of NPS 467 at the Para
thyroid Cell Calcium Receptor

    Dissociated bovine parathyroid cells were used to
further explore the mechanism of action of NPS 467 at
the receptor level. In the presence of 0.5 mM
extracellular Ca^{2+}, NPS R-467 caused a rapid and
transient increase in $[Ca^{2+}]_i$ which persisted in the
presence of 1 μM La^{3+} and was partially depressed by
pretreatment with PMA (100 nM for 2 min.). All these
results are consistent with an action of NPS R-467 on
the Ca^{2+} receptor. However, the cytosolic Ca^{2+}

- 156 -

response to NPS R-467 was abolished when parathyroid
cells were suspended in Ca^{2+}-free buffer. This
suggests that NPS R- 467 cannot, by itself, cause the
mobilization of intracellular Ca^{2+}. It does, however,
elicit responses in parathyroid cells and in oocytes
when a small amount of extracellular Ca^{2+} is present.
This suggests that partial occupancy of the
Ca^{2+}-binding site is required for NPS R-467 to elicit
a response. To test this hypothesis, parathyroid cells
were suspended in Ca^{2+}-free buffer and exposed to a
submaximal concentration of neomycin. Neomycin was used
because it mimics, in nearly all respects, the effects
of extracellular Ca^{2+} on parathyroid cells and on
Xenopus oocytes expressing the parathyroid cell Ca^{2+}
receptor. The addition of 10 àM neomycin did not by
itself cause an increase in $[Ca^{2+}]_i$ under these
conditions. However the subsequent addition of NPS
R-467 (30 μM) now elicited a transient increase in
$[Ca^{2+}]_i$ which, because there was no extracellular
Ca^{2+} present, must have come from the mobilization of
intracellular Ca^{2+}. When cells bathed in Ca^{2+}-free
buffer were exposed to 30 àM NPS R-467 there was no
increase in $[Ca^{2+}]_i$. This concentration of NPS R-
467 is maximally effective in increasing $[Ca^{2+}]_i$
when extracellular Ca^{2+} (0.5 mM) is present. However,
the subsequent addition of 10 μM neomycin now evoked a
transient increase in $[Ca^{2+}]_i$. Presumably, neomycin
binds to the same site as extracellular Ca^{2+} and can
functionally substitute for it. Using a submaximal
concentration, which by itself causes no response,

- 157 -

achieves partial occupancy of the Ca^{2+}-binding site
and allows activation of the Ca^{2+} receptor by NPS
R-467.

Additional studies to further define the
mechanism of action of NPS R-467 were performed. The
cells were once again suspended in Ca^{2+}-free buffer to
insure that any observed increase in $[Ca^{2+}]_i$
resulted from the mobilization of intracellular Ca^{2+}.
In these experiments, however, a maximally effective
concentration (100 μM) of neomycin was used. In the
absence of extracellular Ca^{2+}, 100 μM neomycin
evoked a rapid and transient increase in $[Ca^{2+}]_i$.
The subsequent addition of 30 μM NPS R-467 did not
cause an increase in $[Ca^{2+}]_i$. In the converse
experiment, 30 μM NPS R-467 was added before 100 μM
neomycin. As expected, NPS R-467 did not cause any
increase in $[Ca^{2+}]_i$. It did not, however, affect
the increase in $[Ca^{2+}]_i$ evoked by the subsequent
addition of 100 μM neomycin. These results, obtained
with maximally effective concentrations of NPS R-467 and
neomycin, suggest that these two compounds do not act at
the same site. Rather, the results can be sufficiently
explained by postulating two separate sites on the
Ca^{2+} receptor, one to which extracellular Ca^{2+} and
neomycin bind, and another to which NPS R-467 and
structurally related compounds (such as NPS R-568)
bind. Ligand binding to the former site can result in
full activation of the Ca^{2+} receptor whereas ligand
binding to the latter site can only occur and/or be
functionally relevant when the extracellular

Ca^{2+}-binding site is occupied to some as yet undefined degree. It is possible that ligand binding to the extracellular Ca^{2+}- binding site exposes a previously occluded binding site for NPS R-467. It appears that the NPS R-467-binding site is an allosteric site that augments receptor activation in response to ligand binding at the extracellular Ca^{2+}-binding site.

The data demonstrate that the parathyroid cell Ca^{2+} receptor possesses at least two distinct sites for organic ligands. One site binds the physiological ligand, extracellular Ca^{2+}, and certain organic polycations like neomycin. Binding to this site result in full activation of the Ca^{2+} receptor; an increase in $[Ca^{2+}]_i$, and the inhibition of PTH secretion. NPS R-467 and NPS R-568 define a previously unrecognized binding site on the Ca^{2+} receptor. Binding to this site can only occur and/or results in full activation of the Ca^{2+} receptor when the extracellular Ca^{2+}-binding site is partially occupied. Ligands acting at either site are effective in suppressing serum Ca^{2+} levels in vivo.

Allosteric Site on Parathyroid Cell Calcium Receptor

Calcimimetic compounds that activate the bovine parathyroid cell calcium receptor, such as NPS R-467 and NPS R-568, do not cause the mobilization of intracellular Ca^{2+} in the absence of extracellular Ca^{2+}. Rather, they increase the sensitivity of the Ca^{2+} receptor to activation by extracellular Ca^{2+}, thus causing a shift to the left in the concentration-response curve for extracellular Ca^{2+}. Because of

this, it is unlikely that they act at the same site on
the receptor as does extracellular Ca^{2+}. In contrast,
organic and inorganic polycations do cause the
mobilization of intracellular Ca^{2+} in the absence of
extracellular Ca^{2+} and therefore probably act at the
same site as does extracellular Ca^{2+}. Compounds like
NPS R-568, presumably act in an allosteric manner and
their activity is dependent on some minimal level of
extracellular Ca^{2+}. This suggests that partial
occupancy of the extracellular Ca^{2+}-binding site on
the receptor is required for compounds like NPS R-568 to
be effective. This model is consistent with the
observations described in Example 20.

Other details of the mechanism of action of NPS
R- 568 on the parathyroid cell Ca^{2+} receptor, however,
are more accurately investigated by binding studies in
which the specific binding of radiolabeled (using ^3H
for example) NPS R-568 is assessed. There are several
molecular mechanisms that could explain the activity of
NPS R-568 on the parathyroid cell Ca^{2+} receptor. In
one mechanism (model 1), NPS R-568 could bind to the
Ca^{2+} receptor at a site that, when occupied, is not
sufficient to activate the receptor functionally.
Activation only occurs when some level of occupancy of
the extracellular Ca^{2+}-binding site(s) is achieved.
In an alterative mechanism (model 2), the occupation of
the extracellular Ca^{2+}-binding site could unmask
latent binding sites for compounds such as NPS R-568.
Occupancy of this latent site by NPS R-568 then
increases the affinity and/or efficacy of binding at the

extracellular Ca^{2+} site. Either mechanism involves a
form of allosteric activation of the Ca^{2+} receptor by
compounds such as NPS R-568. These are not the only
possible mechanisms that could explain the effect of
compounds like NPS R-568 on the parathyroid cell Ca^{2+}
receptor. Other mechanisms of action may be suggested
by the results of the binding studies described below.

To further investigate the mechanism of action of
compounds like NPS R-568 on the parathyroid cell Ca^{2+}
receptor, binding studies using ^{3}H-NPS R-568 can be
performed. The specific binding of ^{3}H-NPS R-568 to
intact parathyroid cells or to membranes prepared from
parathyroid cells is initially investigated by
techniques well-known in the art. The kinetic
parameters of binding will then be measured as a
function of extracellular Ca^{2+} concentrations.
Specifically, Scatchard analysis of the data will reveal
the number of binding sites and the apparent affinity of
the receptor site for ^{3}H-NPS R-568. These parameters
will then be investigated as a function of changes in
the level of extracellular Ca^{2+} in the buffer used for
the assay. If model 1 is correct, then a significant
level of specific binding should occur in the absence of
extracellular Ca^{2+}. Large changes in the kinetic
parameters of binding as a function of the level of
extracellular Ca^{2+} would favor model 2. It is
expected that various other inorganic and organic
polycations described above in other examples will cause
similar changes in the binding parameters of ^{3}H-NPS
R-568 as does extracellular Ca^{2+}. This would support

the view that these polycations act at the extracellular
Ca^{2+}-binding site, which is distinct from that to
which compound like NPS R-568 bind.

Example 21:   Preparation of NPS 467

        In a 250 ml round bottom flask, 10.0 g (100
mmoles) 3'-methoxy acetophenone and 13.5 g (100 mmoles)
3-phenylpropylamine were mixed and treated with 125
mmoles (35.5 g) titanium(IV) isopropoxide. The reaction
mixture was stirred 30 minutes·at room temperature under
a nitrogen atmosphere. After this time 6.3 g (100
mmoles) sodium cyanoborohydride in 100 ml ethanol was
added dropwise over the course of 2 minutes. The
reaction was stirred room temperature under nitrogen for
16 hours. After this time the reaction mixture was
transferred to a 2 L separator funnel with 1.5 L ethyl
ether and 0.5 L water. The phases were equilibrated and
the ether layer removed. The remaining aqueous phase
was thoroughly extracted with four 1 L portions of
ether. The washes were combined, dried over anhydrous
potassium carbonate and reduced to a clear, light amber
oil.

        TLC analysis of this material on silica using
chloroform-methanol-isopropylamine (100:5:1) showed
product at R_f 0.65 with traces of the two starting
materials at R_f 0.99 (3'-methoxy acetophenone) and
R_f 0.0 (3-phenylpropylamine).

        The reaction mixture was chromatographed through
silica (48 x 4.6 cm) using a gradient of chloroform-
methanol-isopropylamine (99:1:0.1) to (90:10:0.1) which
yielded 13.66 g of purified NPS 467. This material was

- 162 -

dissolved in hexane-isopropanol (99:1) containing 0.1%
diethylamine to yield a solution with a concentration of
50 mg/ml. Chiral resolution was accomplished by chroma
tography of 4 ml of this solution (200 mg, maximum to
achieve separation) through ChiralCel OD (25 x 2 cm)
using 0.7% isopropylamine, 0.07% diethylamine in hexane
at 100 ml/min, monitoring optical density at 260 nm.
Under these conditions (with injections of 100 mg
material) the early-eluting isomer (NPS 467R) began to
emerge from the column at à26 min, the late eluting
isomer (NPS 467S) began to emerge at 34 minutes.
Baseline resolution was accomplished under these
conditions. Each optical isomer (free base) was
converted to the corresponding hydrochloride salt by
dissolving 3 g of the free base in 100 ml ethanol and
treating with it with 100 ml water containing 10 molar
equivalents HCl. Lyophilization of this solution
yielded a white solid.

NPS 568

NPS 568 is a structural analog of NPS 467 that is
more potent in causing increases in $[Ca^{2+}]_i$ in
bovine and human parathyroid cells. Like NPS 467, the
effects of NPS 568 are stereospecific and it is the
R-isomer that is the more potent enantiomer (see Table
6, supra). NPS R-568 is at present the lead
calcimimetic compound with selective activity at the
parathyroid cell Ca^{2+} receptor. It behaves, albeit
with greater potency, similarly to NPS R-467 as
described in Examples above. Thus, NPS R- 568 evokes
increases in $[Ca^{2+}]_i$ in bovine parathyroid cells in

a stereospecific manner (see Table 6, supra). NPS R-568 fails to evoke increases in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} but it does potentiate responses to extracellular Ca^{2+}. NPS R-568 shifts the concentration-response curve for extracellular Ca^{2+} to the left.

The oral administration of NPS R-568 to rats causes a dose-dependent decrease in the levels of serum Ca^{2+} (ED_{50} = 7mg/kg). The hypocalcemic response elicited by the oral administration of NPS R-568 is rapid in onset and is paralleled by decreases in the levels of serum PTH. The hypocalcemic response evoked by the oral administration of NPS R-568 is only marginally affected by prior complete nephrectomy. However, NPS R-568 fails to elicit a hypocalcemic response in parathyroidectomized rats. NPS R-568 can thus target selectively the parathyroid cell Ca^{2+} receptor in vivo and cause an inhibition of PTH secretion. The decreases in serum levels PTH together with the resulting hypocalcemia are desirable therapeutic effects in cases of hyperparathyroidism. The synthesis of NPS R-568 is provided below.

Example 22:   Preparation of NPS 568

NPS 568 was prepared using the methods described in Example 21 substituting an equivalent amount of 3-(2-chlorophenyl)propylamine for 3-phenylpropylamine. It was found that allowing the mixture of 3'-methoxyacetophenone, 3-(2-chlorophenyl)propylamine and titanium(IV) isopropoxide to stir for 5 hours prior to treatment with $NaCNBH_3$/EtOH resulted in significantly

- 164 -

greater yield (98%).

Example 23:   NPS 467 Lowers Serum Ionized Calcium When
Administered Orally

     Rats (male, Sprague-Dawley, 250-300 g) were fed
standard rat chow and fasted overnight prior to the
experiment. NPS R-467 was suspended in corn oil and
administered as a single oral dose through a gavage
needle. Three hours later a sample of blood was taken
from the tail vein and assessed for ionized Ca^{2+}
levels. Fig. 44 shows that NPS R-467 caused a
dose-dependent reduction in serum levels of ionized
Ca^{2+} when administered orally.

Example 24

  BOPCAR1 Cloning Method 1

     An expression cloning strategy using Xenopus
laevis oocytes was used to identify the cDNA encoding
the bovine parathyroid calcium receptor. These
techniques are described only briefly here, and a more
complete description preceded this one in the sections
describing techniques which may be used to clone
additional forms of the Ca^{2+}-receptor from other cell
types. Poly(A^+)-enriched RNA was initially prepared
from bovine parathyroid glands using extraction with
guanidinium thiocyanate, centrifugation through CsCl and
oligo(dT) cellulose chromatography. Injection of the
resultant poly(A^+)-enriched RNA into oocytes
(25-50 ng/oocyte) conferred sensitivity to elevated
extracellular concentrations of Ca^{2+} and the trivalent
cation (1-100 μM) Gd^{3+} as described herein, such that
the two cations elicited calcium activated chloride

currents. No such currents were elicited in control
eggs injected with water. X. laevis frogs were selected
on the basis of their harvested oocytes exhibiting: (i)
a high level of maturity (i.e., Stage V, VI); (ii) a
high activity of Cl^- currents activated by Ca^{2+}
ionophores like A23187; (iii) a high level of functional
expression of Gd^{3+}-induced Cl^- current when injected
with 25 ng/oocyte of total poly(A)$^+$RNA isolated from
bovine parathyroid. The mRNA was then subjected to size
fractionation, utilizing preparative, continuous flow
agarose gel electrophoresis (Hediger, M.A., Anal.
Biochem. 159:280-286 (1986)) to obtain fractions of
poly(A$^+$)-RNA further enriched in transcripts coding
for the Ca^{2+}-receptor. A peak of activity (e.g.,
oocytes injected with mRNA from these fractions showed
enhanced expression of Gd^{3+}-activated Cl^-currents
obtained with a size range of 4-5.5 kb. This RNA was
used to prepare a size-selected, directional cDNA
library in the plasmid pSPORT1 that was enriched in full
length transcripts. Sense complementary RNA (cRNA) was
then synthesized from the DNA inserts pooled from
350-500 independent clones from this library and
injected into oocytes. Gd^{3+}-activated Cl^-currents
were observed following injection of RNA from a single
filter containing 350 colonies. Preparation and
injection of cRNA from successively smaller pools of
clones led to isolation of a single clone (BoPCaR 1)
with a cDNA insert of 5.3 kb which expressed greatly
enhanced Ca^{2+}-receptor activity following injection of
its cRNA into oocytes. A plasmid containing the BoPCaR

1 cRNA (See plasmid, Figure 45; restriction map, Figure 46;
and partial nucleotide sequence, Figure 47) has been
deposited in the ATCC under deposit number ATCC 75416.

The BoPCaR 1 cDNA is outside the size range of the
size-selected RNA found to express neomycin elicited Cl^-
channel activity in Xenopus oocytes. This is consistent
with the possibility that different isoforms or multiple
genes may conceal other numbers of the Ca^{2+} receptor gene
family.

Several pharmacological and biochemical criteria were
used to identify this clone as encoding a bona fide bovine
parathyroid Ca^{2+}-receptor. Oocytes expressing the cloned
receptor, but not water-injected oocytes, responded to
increasing concentrations of extracellular Ca^{2+} (1.5-5 mM)
or Gd^{3+} (20-600μM) with large increases in Cl^-
currents (up to at least 1.8 microamperes) that were several
fold larger than those observed in poly(A^+)-injected
oocytes. These responses increased markedly over a period
of one (1) to four (4) days after injection of the eggs with
cRNA prepared from the BoPCaR 1 cDNA. Furthermore, the
ranges of the concentrations of the two cations eliciting
this response were very similar to those shown previously to
act on bovine parathyroid cells in vitroN   eomycin
(20-100 μM), which is known to mimic closely the effects
of Ca^{2+} on parathyroid cells, produced changes in Cl^-
current in oocytes essentially identical to those produced
by Ca^{2+} or Gd^{3+}, and these occurred over the same range
of concentrations over which this antibiotic moculates
parathyroid

- 167 -

function in vitro. Finally, in vitro translation of RNA
prepared from the clone resulted in a single major
protein on polyacrylamide gels with a molecular weight
of about 100 kd, whose synthesis was enhanced by
inclusion of dog pancreatic microsomes, concomitant with
an increase in apparent molecular weight of 10-15%. The
latter suggests that the cloned receptor interacts
strongly with membranes, as might be expected of an
integral membrane protein receptor, and is glycosylated
in its native form. Studies with the lectin
concanavalin A indicate that the Ca^{2+}-receptor is
likely a glycoprotein. Thus, the pharmacological
properties of the cloned receptor, which is expressed at
high levels in oocytes, as well as the biochemical
studies carried out to date are completely consistent
with its identity with the bovine parathyroid Ca^{2+}
dynamics (as exemplified by changes in Ca^{2+}-activated
Cl^- currents in oocytes).

Diagnostic Use

NPS R-568 or other compounds active on a calcium
receptor can be used as a diagnostic tool.
Specifically, a pharmaceutical preparation of such
compounds is useful as a diagnostic tool. In one
example, a pharmaceutical preparation containing a para
thyroid cell calcimimetic compound such as NPS R-568 can
be given by oral or another route of administration to
hypercalcemic patients with symptoms of mental
depression. If these symptoms arise from an underlying
hyperparathyroid state, such as primary hyper
parathyroidism, then administration of NPS R-568 or a

compound that acts similarly will alleviate those
symptoms. If the symptoms do not abate, then the mental
depression results from some pathological state that is
not hyperparathyroidism. Thus, parathyroid cell
calcimimetic compounds can be used in the differential
diagnosis of mental depression.

Symptoms and signs common to hyperparathyroidism
and other disorders can also be differentially diagnosed
in the manner described above. Such shared signs and
symptoms include, but are not limited to, hypertension,
muscular weakness, and a general feeling of malaise.
Alleviation of these symptoms following treatment with a
parathyroid cell Ca^{2+} receptor calcimimetic compound
would indicate that the problems result from the
underlying hyperparathyroidism.

In another example, a compound acting as an
antagonist (calcilytic) at the C-cell Ca^{2+} receptor
can be administered as described above to diagnose
medullary thyroid carcinoma. In this case,
administration of the C-cell Ca^{2+} receptor calcilytic
compound will depress serum levels of calcitonin which
can be readily measured by radioimmunoassay. Certain
symptoms associated with medullary thyroid carcinoma,
such as diarrhea, may also be monitored to determine if
they are abated or lessened following administration of
the calcilytic compound.

In a third example, a compound acting as a
calcimimetic at the juxtaglomerular cell Ca^{2+} receptor
can be used in the differential diagnosis of
hypertension. In this case, administration of the

juxtaglomerular cell Ca^{2+} receptor calcimimetic
compound can be administered as described above. A
decrease in blood pressure to normal levels will occur
if the hypertension results mostly or exclusively from
elevated levels of renin rather than from an alternative
pathological state.

In another example, a compound acting as a
specific calcimimetic on the osteoclast Ca^{2+} receptor
can be used in the differential diagnosis of high- and
low- turnover forms of osteoporosis. In this case, such
a compound can be administered in a suitable
pharmaceutical preparation and the levels of serum
alkaline phosphatase, osteocalcin, pyridinoline and/or
deoxypyridinoline crosslinks, and/or some other
predictive marker of bone resorption and/or formation
measured by techniques well known in the art. A large
decrease in one or more of these parameters would be
predictive of high-turnover osteoporosis, whereas a
small or no decrease in these parameters would be
predictive of low-turnover osteoporosis. Such
information would dictate the appropriate treatment.
Antiresorptive drugs would not be the appropriate sole
therapy for low-turnover osteoporosis.

These examples are not exhaustive but serve to
illustrate that specific Ca^{2+} receptors can be
targeted with pharmaceutical preparations and that the
observed effects of such preparations on bodily
functions and/or chemical constituents can be used
diagnostically. In general, calcimimetic and calcilytic
compounds that act on Ca^{2+} receptors of the various

cells described above can be used in the diagnosis of
the various diseases associated with the particular cell
type. These diseases include but are not limited to
bone and mineral-related disorders (as described in Coe
et al., Disorders of Bone and Mineral Metabolism, Raven
Press, 1990), kidney diseases, endocrine diseases,
cancer, cardiovascular diseases, neurological diseases,
gastrointestinal diseases, and diseases associated with
gestation. Examples of human diseases or disorders in
which such molecules may be therapeutically effective
are as follows: (1) a calcimimetic is expected to
ameliorate psoriasis by reducing the proliferation of
the abnormal skin cells. (2) Since Ca^{2++} blocks the
effect of vasopressin on MTAL and cortical collecting
cells, a calcimimetic is expected to reduce water
retention in states of vasoprsssin excess, such as the
syndrome of inappropriate vasopressin (ADH) secretion.
Conversely, calcium receptor antagonists used in states
of ADH deficiency are expected to potentiate the action
of any ADH present, such as in partial central diabetes
insipidus. (3) Calcimimetics may be used to treat
hypertension by (a) reducing renin secretion, (b) by
stimulating production of vasodilators such as PTHrP
(PTH-related peptide) by vascular smooth muscle. (4)
Calcimimetics are expected to increase platelet
aggregability, which may be useful when platelet counts
are low. Conversely, calcilytics are expected to
inhibit platelet function in states where there is
hypercoagulability. (5) Calcium promotes
differentiation of colon and mammary cells. A

- 171 -

calcimimetic is expected to reduce the risk of colon or
breast cancer. (6) Calcium promotes urinary calcium
excretion in the MTAL. A calcimimetic is expected to
have a useful hypocalcemic action in the therapy of
hypercalcemic disorders. The inhibitory effect of
calcimimetics on osteoclasts and their stimulation of
the secretion of the hypocalcemic peptide calcitonin
make them expected to be useful in the therapy of
hypercalcemia and its symptoms. A calcimimetic may also
improve hypocalcemic symptoms by activating calcium
receptors. Conversely, calcilytic is expected to reduce
urinary calcium excretion and be useful in the treatment
of kidney stones. In addition, calcium suppresses the
formation of 1,25-dihydroxyvitamin D in the proximal
renal tubule, and this vitamin D metabolite is
frequently overproduced in renal stone patients and
contributes to their hypercalciuria. Supression of
1,25-dihydroxyvitamin D formation by a calcimimetic is
expected to be useful treating renal calcium stone
disease. (7) Endogenous amines could reproduce the
symptoms in uremic patients by calcimimetic or lytic
actions. Calcimimetic and/or calcilytic agents are
expected to improve these symptoms. (8) Some of the
renal toxicity of aminoglycoside antibiotics may be
mediated by interaction of these drugs with renal
calcium receptors. Having the calcium receptor is
expected to make it possible to carry out drug screening
easily when designing new drugs of these classes to
minimize renal toxicity. In addition, a renal calcium
receptor antagonist would prevent or treat this renal

- 172 -

toxicity if it is related to this mechanism. (9) Some
of the genetic component of calcium related disorders,
such as osteoporosis, renal stones, and hypertension are
expected to be related to inherited problems with
certain forms of the receptor. These now can be studied
and genetic screening/testing carried out using
receptor-based reagents. The human disease, familial
hypocalciuric hypercalcemia, may be due to a calcium
receptor defect. Definitive diagnostic separation from
cases of promary hyperparathyroidism could be carried
out with receptor-based technology. (10) Calcium
receptors are present in the placenta and are expected
to impact on disorders of placental function and
transfer of nutrients to the growing fetus.

Other embodiments are within the following claims.

We Claim:

8907V

<u>SEQUENCE LISTING</u>

(1) GENERAL INFORMATION:

    (i)   APPLICANT: Nemeth, Edward F.

    (ii)   TITLE OF INVENTION: CALCIUM RECEPTOR-ACTIVE
           MOLECULES

    (iii) NUMBER OF SEQUENCES: 1

    (iv)   CORRESPONDENCE ADDRESS:
           (A)   ADDRESSEE: Wolf, Greenfield & Sacks, P.C.
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           (D)   STATE: Massachusetts
           (E)   COUNTRY: United States of America
           (F)   ZIP: 02210-2204

    (v)   COMPUTER READABLE FORM:
           (A)   MEDIUM TYPE: Diskette, 5.25 inch
           (B)   COMPUTER: IBM-compatible
           (C)   OPERATING SYSTEM: MS:DOS Version 3.3
           (D)   SOFTWARE: WordPerfect 5.1

    (vi)   CURRENT APPLICATION DATA:
           (A)   APPLICATION NUMBER: Not Available
           (B)   FILING DATE: Filed Herewith
           (C)   CLASSIFICATION: Not Available

    (viii) ATTORNEY/AGENT INFORMATION:
           (A)   NAME: Gates, Edward R.
           (B)   REGISTRATION NUMBER: 31,616
           (C)   REFERENCE/DOCKET NUMBER: B0801/7012

```
(ix)   TELECOMMUNICATION INFORMATION:
       (A)   TELEPHONE:  (617) 720-3500
       (B)   TELEFAX:  (617) 720-2441
       (C)   TELEX:  EZEKIEL


(2) INFORMATION FOR SEQ ID NO: 1:
    (i)    SEQUENCE CHARACTERISTICS:
           (A)   LENGTH:  2148 base pairs
           (B)   TYPE:  nucleic acid
           (C)   STRANDEDNESS:  double
           (D)   TOPOLOGY:  linear


    (ii)   MOLECULE TYPE:  cDNA to mRNA


    (iii)  HYPOTHETICAL:  No


    (iv)   ANTI-SENSE:  No


    (xi)   SEQUENCE DESCRIPTION:  SEQ ID NO: 1:
    CCACGCGTCC  GCGGAAAAAA  AAAAAAAGTT  CCCCACTCTA      40
    GTACAGAGAA  GGTTGGCAGA  GTCGTAAGCC  CCCAACCTCT      80
    TAAACTTCTC  TGCATCTCCA  AGGAGAAGGA  GGGAAGAGGG     120
    GTTCTTTCCG  ACCTGAGGAG  CTGGATCTGG  GGTCCGAGAA     160
    CCCCAAGGTA  GCACCGGAAA  GAACAGCACA  GGAGGCGAGA     200
    GCGTGCGGTG  GCCGGTKGGC  GGGAGAACCA  GACCCGACKY     240
    CGCGGTCCTC  GGYGCTGCCG  GGKYCCSGGG  ACTCAGCTCA     280
    GCACGACTGG  GAAGCCGAAA  GTACTACACA  CGGTCTCTGC     320
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ATGATGTGAC TTCTGAAGAC TCAAGAGCCA CCCACTTCAC    360
TAGTCTGCAA TGGAGAAGGC AGAAATGGAA AGTCAAACCC    400
CACGGTTCCA TTCTATTAAT TCTGTAGACA TGTGCCCCCA    440
CTGCAGGGAG TGAGTCGCAC CAAGGGGGAA AGTCCTCAGG    480
GGCCCCCAGA CCACCAGCGC TTGAGTCCCT CTTCCTGGAG    520
AGAAAGCAGA ACTATGGCAC TTTATAGCTG CTGTTGGATC    560
CTCTTGGCTT TTTTCTACCT GGTGCACTTC CGCCTATGGG    600
CCTGACCAGC GAGCCCAAAA GAAAGGGGAC ATTATCCTCG    640
GGGGGCTCTT TCCTATTCAT TTTGGGGTTG MMGTGAAAGA    680
TCAGGATCTA AAGTCGAGGC CGGAGTCCGT GGAGTGTATC    720
AGGTATAATT TCCGAGGATT TCGCTGGTTA CAAGCTATGA    760
TATTTGCCAT AGAGGAAATA AACAGCAGTC CAGCCCTTCT    800
TCCCAACATG ACCCTGGGAT ACAGGATATT CGACACTTGT    840
AACACCGTCT CTAAAGCCTT GGAGGCCACC CTGAGTTTTG    880
TGGCCCAGAA CAAAATTGAC TCTTTGAACC TTGATGAGTT    920
CTGCAACTGC TCAGAGCACA TCCCCTCTAC CATCGCAGTG    960
GTGGGAGCTA CTGGCTCGGG CATCTCCACA GCAGTGGCCA   1000
ACCTGCTGGG GCTCTTCTAC ATCCCCCAGG TCAGCTATGC   1040
CTCCTCCAGC AGACTCCTCA GCAACAAGAA TCAATTCAAG   1080
TCCTTCCTCC GCACCATACC CAATGATGAA CACCAGGCCA   1120
CGGCCATGGC TGACATCATC GAGTACTTCC GCTGGAACTG   1160
GGTGGGCACA ATTGCAGCTG ACGATGACTA TGGCCGGCCA   1200
GGGATCGAGA AGTTTCGAGA GGAAGCTGAG GAGAGGGACA   1240
TCTGCATCGA CTTCAGCGAG CTCATCTCCC AATACTCTGA   1280
TGAGGAAAAG ATCCAGCAGG TGGTGGAGGT GATCCAGAAT   1320
TCCACCGCCA AAGTCATTGT CCGTCTTCTC CAGCGGCCCA   1360
GACCTGGAAC CCCTCATCAA AGAGATCGTC CGGCGCAATA   1400
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- 176 -

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TCACAGGCAG GATCTGGCTG GCCAGCGAGS CTGGSCAGCT    1440
CTTCCCTGAT TGCTATGCCC GAGTATTTCC ATGTGGTCGG    1480
AGGCACCATT GGGTTTGGTT TGAAAGCTGG GCAGATCCCA    1520
GGCTTCCGGG AATTCCTGCA GAAAGTCCAC CCCAGGAAGT    1560
CTGTCCACAA TGGTTTTGCC AAGGAGTTTT GGGAAGAAAC    1600
ATTTAACTGC CACCTGCAAG AGGGTGCTAA AGGCCCATTA    1640
CCGGTGGACA CCTTCCTGAG AGGTCACGAA GAAGGAGGTG    1680
CCAGGTTAAG CAACAGTCCC ACTGCCTTCC GACCTCTGTG    1720
CACTGGGGAG GAGAACATCA GCAGTGTCGA GACTCCTTAC    1760
ATGGATTATA CACATTTACG GATATCCTAC AACGTCTACT    1800
TAGCCGTCTA CTCCATTGCT CATGCCCTAC AAGATATATA    1840
CACCTGCATA CCTGGGAGAG GGCTCTTCAC CAACGGTTCC    1880
TGCGCAGATA TCAAGAAGGT TGAAGCTTGG CAGGTCCTGA    1920
AACACCTGCG GCACCTAAAT TTTACCAGCA ATATGGGGGA    1960
GCAAGTAACT TTCGATGAAT GTGGAGACCT GGCAGGGAAC    2000
TATTCCATCA TCAACTGGCA CCTCTCCCCA GAGGACGGCT    2040
CCATAGTGTT TAAGGAAGTT GGATATTACA ATGTCTATGC    2080
CAAGAAAGGA GAGAGACTCT TCATCAATGA TGAAAAAATT    2120
CTGTGGAGTG GATTCTCAAG GGAGGTGC                 2148
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8907V

- 177 -

We Claim:

1.   A pharmaceutical composition comprising
a molecule which mimics the activity of extracellular
Ca^{2+} by evoking an increase in $[Ca^{2+}]_i$ in a cell, or
blocks an increase in $[Ca^{2+}]_i$ evoked by extracellular
Ca^{2+}, said molecule having an EC_{50} of less than or equal
to 5 μM, wherein said molecule is not protamine.

2.   The pharmaceutical composition of claim 1
wherein said increase in $[Ca^{2+}]_i$ in a cell is transient,
having a duration of less than thirty seconds.

3.   The pharmaceutical composition of claim 1
or 2 wherein said increase in $[Ca^{2+}]_i$ is rapid, occurring
within thirty seconds.

4.   The pharmaceutical composition of claim 1
wherein said molecule evokes a sustained increase in
$[Ca^{2+}]_i$, having a duration greater than thirty seconds.

5.   The pharmaceutical composition of claim 1
wherein said molecule further evokes an increase in
inositol-1,4,5-trisphosphate or diacylglycerol.

6.   The pharmaceutical composition of claim 5
wherein said increase in said inositol-1,4,5-
trisphosphate or diacylglycerol occurs within 60
seconds.

7.   The pharmaceutical composition of claim 2
wherein said transient increase is diminished by
pretreatment of said cell for less than 10 minutes with
an activator of protein kinase C.

8.   The pharmaceutical composition of claim 7
wherein said activator of protein kinase C is selected

from the group consisting of phorbol myristate acetate,
mezerein and (-)-indolactam V.

9.   The pharmaceutical composition of claim 1
wherein said molecule inhibits dopamine- or
isoproterenol-stimulated cyclic AMP formation.

10.   The pharmaceutical composition of claim
2 wherein said transient increase in $[Ca^{2+}]_i$ is reduced
by pretreatment of said cell for ten minutes with 10 mM
sodium fluoride.

11.   The pharmaceutical composition of claim
1 wherein said cell is a parathyroid cell and said
molecule inhibits parathyroid hormone secretion from
said cell.

12.   The pharmaceutical composition of claim
1 wherein said molecule elicits an increase in Cl^-
conductance in a <u>Xenopus</u> oocyte injected with mRNA from
a cell selected from the group consisting of parathyroid
cells, bone osteoclasts, juxtaglomerular kidney cells,
proximal tubule kidney cells, keratinocytes,
perifollicular thyroid cells and placental trophoblasts.

13.   The pharmaceutical composition of claim
1 wherein said molecule mobilizes intracellular Ca^{2+} to
cause said increase in $[Ca^{2+}]_i$.

14.   The pharmaceutical composition of claim
1 wherein said cell is a C-cell or an osteoclast and
said molecule inhibits bone resorption <u>in vivo</u>.

15.   The pharmaceutical composition of claim
1 wherein said cell is a C-cell and said molecule
stimulates calcitonin secretion <u>in vitro</u> or <u>in vivo</u>.

16. The pharmaceutical composition of claim 1 wherein said molecule is either a calcimimetic or calcilytic having an EC_{50} or IC_{50} at a Ca^{2+} receptor of less than or equal to 5 μM.

17. The pharmaceutical composition of claim 1 wherein said molecule has an EC_{50} less than or equal to 5 μM at one or more but not all cells chosen from the group consisting of parathyroid cells, bone osteoclasts, juxtaglomerular kidney cells, proximal tubule kidney cells, keratinocytes, perifollicular thyroid cells and placental trophoblasts.

18. The pharmaceutical composition of claim 1 or 16 or 17 wherein said molecule has an EC_{50} or IC_{50} less than or equal to 1 μM.

19. The pharmaceutical composition of claim 1 or 16, or 17 wherein said molecule has an EC_{50} or IC_{50} less than or equal to 100 nanomolar.

20. The pharmaceutical composition of claim 1 or 16, or 17 wherein said molecule has an EC_{50} or IC_{50} less than or equal to 10 nanomolar.

21. The pharmaceutical composition of claim 1 or 16, wherein said molecule has an EC_{50} or IC_{50} less than or equal to 1 nanomolar.

22. The pharmaceutical composition of claim 1, 16 or 17 wherein said molecule is positively charged at physiological pH.

23. The pharmaceutical composition of claim 22 wherein said molecule is selected from the group consisting of branched or cyclic polyamines, positively charged polyamino acids, and arylalkylamines.

24. The pharmaceutical composition of claim 23 wherein said branched polyamine has the formula
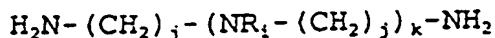
$$H_2N-(CH_2)_j-(NR_i-(CH_2)_j)_k-NH_2$$

wherein k is an integer from 1 to 10,

each j is the same or different and is an integer from 2 to 20, and

each R_i is the same or different and is selected from the group consisting of hydrogen and $-(CH_2)_j-NH_2$, wherein j is as defined above, and wherein at least one R_i is not hydrogen.

25. The pharmaceutical composition of claim 1, 16 or 17 wherein said molecule has the formula
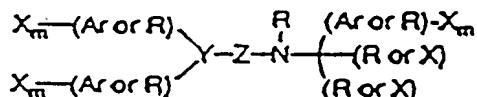
wherein each X is selected (independently) from the group consisting of H, CH_3, CH_3O, CH_3CH_2O, Br, Cl, F, CF_3, CHF_2, CH_2F, CF_3O, CH_3S, OH, CH_2OH, $CONH_2$, CN-, NO_2, and CH_3CH_2;   Ar is a hydrophobic entity;

each R independently is selected from the group consisting of hydrogen, methyl, ethyl, propyl, isopropyl, butyl, isobutyl, cyclopentyl, cyclohexyl, cycloheptyl, cyclooctyl, indenyl, indanyl, dihydroindolyl, thiodihydroindolyl, and 2-,3-, or 4-piperid(in)yl;

Y is selected from the group consisting of CH, nitrogen and an unsaturated carbon;

Z is selected from the group consisting of oxygen, nitrogen, sulfur,

where each n is independently between 1 and 4 inclusive,
and each m is independently between 0 and 5 inclusive;
          wherein said molecule is either a
calcimimetic or calcilytic.

          26. The pharmaceutical composition of claim
25 wherein said hydrophobic entity is selected from the
group consisting of phenyl, 2-, 3-, or 4-pyridyl, 1- or
2-naphthyl, 1- or 2-quinolinyl, 2- or 3-indolyl, benzyl,
and phenoxy.

          27. A pharmaceutical composition comprising
a molecule having the formula

$$X_m-(Ar\ or\ R) \diagdown \qquad \underset{\underset{}{\overset{R}{|}}}{} \quad (Ar\ or\ R)-X_m$$
$$\diagup Y-Z-N-\!\!\!\!\diagdown\!-(R\ or\ X)$$
$$X_m-(Ar\ or\ R)\diagup \qquad\qquad (R\ or\ X)$$

wherein each X independently is selected from the group
consisting of H, CH_3, CH_3O, CH_3CH_2O, Br, Cl, F, CF_3, CHF_2,
CH_2F, CF_3O, CH_3S, OH, CH_2OH, $CONH_2$, CN, NO_2, and CH_3CH_2;
Ar is a hydrophobic entity;
          each R independently is selected from the
group consisting of hydrogen, methyl, ethyl, propyl,
isopropyl, butyl, isobutyl, cyclopentyl, cyclohexyl,
cycloheptyl, cyclooctyl, indenyl, indanyl,
dihydroindolyl, thiodihydroindolyl, and 2-,3-, or 4-
piperid(in)yl;
          Y is selected from the group consisting of
CH, nitrogen and an unsaturated carbon;
          Z is selected from the group consisting of
oxygen, nitrogen sulfur,

$$\underset{\underset{X\ or\ R}{|}}{\overset{\overset{X\ or\ R}{|}}{C}} - \underset{\underset{X\ or\ R}{|}}{\overset{\overset{X\ or\ R}{|}}{C}}\ ,\quad \underset{\underset{X\ or\ R}{|}}{\overset{\overset{X\ or\ R}{|}}{C}} - \left(\underset{\underset{X\ or\ R}{|}}{\overset{\overset{X\ or\ R}{|}}{C}} - \right)_n \underset{\underset{X\ or\ R}{|}}{\overset{\overset{X\ or\ R}{|}}{C}}\ ,\quad \underset{\underset{X\ or\ R}{|}}{\overset{\overset{X\ or\ R}{|}}{C}}\quad and\ CH-(CH_2)_n-$$

where each n is independently between 1 and 4 inclusive,
and each m is independently between 0 and 5 inclusive;

- 182 -

wherein said molecule is not NPS 447 or NPS
449.

28. The pharmaceutical composition of claim
27 wherein said molecule is NPS 467.

29. The pharmaceutical composition of claim
25 wherein said molecule is NPS 459, NPS 467, or NPS
568.

30. The pharmaceutical composition of claim
25, wherein said molecule is an R-phenylpropyl-α-phenet-
hylamine derivative.

31. The pharmaceutical composition of claim
27, wherein said molecule is an R-phenylpropyl-α-phenet-
hylamine derivative.

32. The pharmaceutical composition of claim
30 wherein said molecule has the formula:
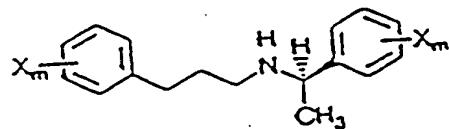
33. The pharmaceutical composition of claim
31 wherein said molecule has the formula:
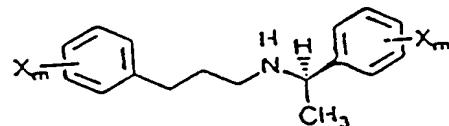
34. The pharmaceutical composition of claim
32 wherein each X is independently selected from the
group consisting of Cl, F, CF_3, CH_3, and CH_3O.

- 183 -

35. The pharmaceutical composition of claim 33 wherein each X is independently selected from the group consisting of Cl, F, CF_3, CH_3, and CH_3O.

36. A method for treating a patient having a disease characterized by an abnormal $[Ca^{2+}]$ or $[Ca^{2+}]_i$ in one or more cells or in the blood or plasma, comprising the step of administering to said patient a therapeutically effective amount of a molecule which mimics the activity of extracellular Ca^{2+} by evoking an increase in $[Ca^{2+}]_i$ in a cell, or blocks an increase in $[Ca^{2+}]_i$ evoked by extracellular Ca^{2+}.

37. The method of claim 36 wherein said molecule has an EC_{50} of less than or equal or 5 μM.

38. The method of claim 36 wherein said molecule is not protamine.

39. The method of claim 36 wherein said molecule interacts at a Ca^{2+} receptor as a calcimimetic or calcilytic.

40. The method of claim 36 wherein said increase in $[Ca^{2+}]_i$ in a cell is transient, having a duration of less than thirty seconds.

41. The method of claim 36 or 40 wherein said increase in $[Ca^{2+}]_i$ is rapid, occurring within thirty seconds.

42. The method of claim 36 wherein said molecule evokes a sustained increase in $[Ca^{2+}]_i$, having a duration greater than thirty seconds.

- 184 -

43.   The method of claim 36 wherein said
molecule further evokes an increase in inositol-1,4,5-
trisphosphate or diacylglycerol.

44.   The method of claim 43 wherein said
increase in said inositol-1,4,5-trisphosphate or
diacylglycerol occurs within less than 60 seconds.

45.   The method of claim 40 wherein said
transient increase is diminished by pretreatment of said
cell with an activator of protein kinase C.

46.   The method of claim 45 wherein said
activator of protein kinase C is selected from the group
consisting of phorbol myristate acetate, mezerein and
(-)-indolactam V.

47.   The method of claim 36 wherein said
molecule inhibits dopamine- or isoproterenol-stimulated
cyclic AMP formation.

48.   The method of claim 40 wherein said
transient increase in $[Ca^{2+}]_i$ is reduced by pretreatment
of said cell for ten minutes with 10 mM sodium fluoride.

49.   The method of claim 36 wherein said cell
is a parathyroid cell and said molecule inhibits
parathyroid hormone secretion from said cell.

50.   The method of claim 36 wherein said
molecule elicits an increase in Cl⁻ conductance in a
Xenopus oocyte injected with mRNA from a cell selected
from the group consisting of parathyroid cells, bone
osteoclasts, juxtaglomerular kidney cells, proximal
tubule kidney cells, keratinocytes, perifollicular
thyroid cells and placental trophoblasts.

51. The method of claim 36 wherein said molecule mobilizes Ca^{2+} within the cell to cause said increase in $[Ca^{2+}]_i$.

52. The method of claim 36 wherein said cell is a C-cell or an osteoclast and said molecule inhibits bone resorption _in vivo_.

53. The method of claim 36 wherein said cell is a C-cell and said molecule stimulates calcitonin secretion _in vitro_ or _in vivo_.

54. The method of claim 36 wherein said molecule is either a calcimimetic or calcilytic having an EC_{50} or IC_{50} at a Ca^{2+} receptor of less than or equal to 5 μM.

55. The method of claim 36 wherein said molecule has an EC_{50} less than or equal to 5 μM at one or more but not all cells chosen from the group consisting of parathyroid cells, bone osteoclasts, juxtaglomerular kidney cells, proximal tubule kidney cells, keratinocytes, perifollicular thyroid cells and placental trophoblasts.

56. The method of claim 36, 54 or 55 wherein said molecule has an EC_{50} or IC_{50} less than or equal to 1 μM.

57. The method of claim 36, 54 or 55 wherein said molecule has an EC_{50} or IC_{50} less than or equal to 100 nanomolar.

58. The method of claim 36, 54, or 55 wherein said molecule has an EC_{50} or IC_{50} less than or equal to 10 nanomolar.

59. The method of claim 38, 54 or 55, wherein said molecule has an EC_{50} or IC_{50} less than or equal to 1 nanomolar.

60. The method of claim 36, 54 or 55 wherein said molecule is positively charged at physiological pH.

61. The method of claim 60 wherein said molecule is selected from the group consisting of branched or cyclic polyalkylamines, positively charged polyamino acids, and arylalkylamines.

62. The method of claim 61 wherein said branched polyamine has the formula

$$H_2N-(CH_2)_j-(NR_i-(CH_2)_j)_k-NH_2$$

wherein k is an integer from 1 to 10, each j is the same or different and is an integer from 2 to 20, and each R_i is the same or different and is selected from the group consisting of hydrogen and $-(CH_2)_j-NH_2$, wherein j is as defined above, and wherein at least one R_i is not hydrogen.

63. The method of claim 36, 54 or 55 wherein said molecule has the formula

wherein each X independently is selected from the group consisting of H, CH_3, CH_3O, CH_3CH_2O, Br, Cl, F, CF_3, CHF_2, CH_2F, CF_3O, CH_3S, OH, CH_2OH, $CONH_2$, CN, NO_2, and CH_3CH_2;

Ar is a hydrophobic entity;

each R independently is selected from the group consisting of hydrogen, methyl, ethyl, propyl, isopropyl, butyl, isobutyl, cyclopentyl, cyclohexyl,

cycloheptyl, cyclooctyl, indenyl, indanyl, dihydroindolyl, thiodihydroindolyl, and 2-,3-, or 4-piperid(in)yl;

Y is selected from the group consisting of CH, nitrogen and an unsaturated carbon;

Z is selected from the group consisting of oxygen, nitrogen, sulfur,

$$
\begin{array}{c}
X\ or\ R \\ | \\ C \\ | \\ X\ or\ R
\end{array}
\begin{array}{c}
X\ or\ R \\ | \\ -\ C \\ | \\ X\ or\ R
\end{array}
\ ,\
\begin{array}{c}
X\ or\ R \\ | \\ C \\ | \\ X\ or\ R
\end{array}
\left(
\begin{array}{c}
X\ or\ R \\ | \\ -\ C\ - \\ | \\ X\ or\ R
\end{array}
\right)_n
\begin{array}{c}
X\ or\ R \\ | \\ C \\ | \\ X\ or\ R
\end{array}
\ ,\
\begin{array}{c}
X\ or\ R \\ | \\ C \\ | \\ X\ or\ R
\end{array}
\ and\
\begin{array}{c}
X\ or\ R \\ | \\ CH- (CH_2)_n-
\end{array}
$$

where each n is independently between 1 and 4 inclusive, and each m is independently between 0 and 5 inclusive; wherein said molecule is either a calcimimetic or calcilytic.

64. The method of claim 63 wherein said hydrophobic entity is selected from the group consisting of phenyl, 2- 3-, or 4-pyridyl, 1- or 2-naphthyl, 1- or 2-quinolinyl, 2- or 3-indolyl, benzyl, and phenoxy.

65. The method of claim 36, 54 or 55 wherein said molecule has the formula

$$
\begin{array}{c}
X_m-(Ar\ or\ R) \\ \\ X_m-(Ar\ or\ R)
\end{array}
Y-Z-N
\begin{array}{c}
R\ \ (Ar\ or\ R)-X_m \\ | \\ -(R\ or\ X) \\ (R\ or\ X)
\end{array}
$$

wherein each X independently is selected from the group consisting of H, CH_3, CH_3O, CH_3CH_2O, Br, Cl, F, CF_3, CHF_2, CH_2F, CF_3O, CH_3S, OH, CH_2OH, $CONH_2$, CN, NO_2, and CH_3CH_2;

Ar is a hydrophobic entity;

each R independently is selected from the group consisting of hydrogen, methyl, ethyl, propyl, isopropyl, butyl, isobutyl, cyclopentyl, cyclohexyl,

cycloheptyl, cyclooctyl, indenyl, indanyl,
dihydroindolyl, thiodihydroindolyl, and 2-,3-, or 4-
piperid(in)yl;

   Y is selected from the group consisting of
CH, nitrogen and an unsaturated carbon;

   Z is selected from the group consisting of
oxygen, nitrogen, sulfur,

$$\begin{matrix} X\ or\ R & X\ or\ R \\ | & | \\ C & - & C \\ | & | \\ X\ or\ R & X\ or\ R \end{matrix} \quad , \quad \begin{matrix} X\ or\ R & X\ or\ R \\ | & | \\ C & - \Bigg(C & - \\ | & | \\ X\ or\ R & X\ or\ R \Bigg)_n \end{matrix} \begin{matrix} X\ or\ R \\ | \\ C \\ | \\ X\ or\ R \end{matrix} \quad , \quad \begin{matrix} X\ or\ R \\ | \\ C \\ | \\ X\ or\ R \end{matrix} \quad and \quad CH-(CH_2)_n-$$

where each n is independently between 1 and 4 inclusive,
and each m is independently between 0 and 5 inclusive;
   wherein said molecule is not NPS 447 or NPS
449.

   66.   The method of claim 65 wherein said
molecule is NPS 467 or NPS 019.

   67.   The method of claim 63 wherein said
molecule is NPS 467 and NPS 568.

   68.   The method of claim 63, wherein said
molecule is an R-phenylpropyl-α-phenethylamine deriva-
tive.

   69.   The method of claim 65, wherein said
molecule is an R-phenylpropyl-α-phenethylamine
derivative.

   70.   The method of claim 68 wherein said
molecule has the formula:
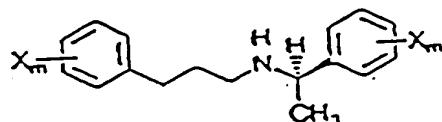
71. The method of claim 69 wherein said molecule has the formula:
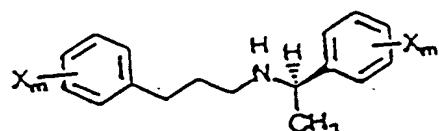
72. The method of claim 70 wherein each X is independently selected from the group consisting of Cl, F, CF$_3$, CH$_3$, and CH$_3$O.

73. The method of claim 71 wherein each X is independently selected from the group consisting of Cl, F, CF$_3$, CH$_3$, and CH$_3$O.

74. The method of claim 36 wherein said patient has a disease characterized by an abnormal level of one or more ions or substances, the level of which is regulated or affected by activity of one or more Ca^{2+} receptors.

75. The method of claim 74, wherein said molecule is active on a Ca^{2+} receptor of a cell selected from the group consisting of parathyroid cells, bone osteoclasts, juxtaglomerular kidney cells, proximal tubule kidney cells, keratinocytes, perifollicular thyroid cells, and placental trophoblasts.

76. The method of claim 36, wherein said patient has a disease selected from the group consisting of primary and secondary hyperparathyroidism, Paget's disease, hypercalcemia malignancy, osteoporosis and hypertension.

77. The method of claim 75 wherein said molecule is active only at one or more said Ca^{2+} receptors and not all said Ca^{2+} receptors.

78. The method of claim 36 wherein said molecule reduces the level of parathyroid hormone in the serum of said patient.

79. The method of claim 78, wherein said level of parathyroid hormone is reduced to that level present in a normal individual.

80. The method of claim 78 wherein said level is reduced to a degree sufficient to cause a decrease in plasma Ca^{2+}.

81. The method of claim 78 wherein said molecule is provided in an amount sufficient to have a therapeutically relevant effect on said patient.

82. The method of claim 36 wherein said molecule blocks an increase in $[Ca^{2+}]_i$ within the cell evoked by extracellular $[Ca^{2+}]$.

83. A method for diagnosis of a disease or condition in a patient, comprising the steps of identifying the number and/or location of one or more Ca^{2+} receptors within said patient and comparing said number and/or location with that observed in normal patients as an indication of the presence of a said disease or condition.

84. The method of claim 83 wherein said method is an immunoassay in which an antibody to a Ca^{2+} receptor is used to identify the number or location of said Ca^{2+} receptors.

-191-

85.   The method of claim 83 wherein said assay comprises providing a labeled calcimimetic or calcilytic molecule which binds to a Ca^{2+} receptor.

86.   The method of claim 83 wherein said disease is a cancer.

87.   The method of claim 86 wherein said cancer is an ectopic tumor in the parathyroid.

88.   The method of claim 83 wherein said condition is characterized by an increased number or activity of the osteoclasts in bone.

89.   A method for treatment of ostioporosis, hyperparathyroidism or hypertension, comprising the steps of administering a therapeutically effective amount of a compound which mimics the activity of extracellular Ca^{2+} by evoking an increase in $[Ca^{2+}]_i$ in a cell, or blocks an increase in $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+}.

90.   A method for identifying a compound useful as a therapeutic agent, comprising the steps of screening a potentially useful molecule for its ability to mimic the activity of extracellular Ca^{2+} in a cell, or to block an increase in $[Ca^{2+}]_i$ (elicited by extracellular Ca^{2+}), and determining whether said molecule has EC_{50} of less than or equal to 5 µM.

SUBSTITUTE SHEET

-192-

91.   A recombinant Ca^{2+} receptor.

92.   A cell comprising a recombinant Ca^{2+} receptor.

93.   A method for identifying a useful calcimimetic compound comprising the step of identifying a compound which mimics one or more activities of Ca^{2+} at a first Ca^{2+} receptor but not at a second Ca^{2+} receptor.

94.   A method for identifying a useful calcilytic compound comprising the step of identifying a compound which blocks one or more activities of Ca^{2+} at a first Ca^{2+} receptor but not at a second Ca^{2+} receptor.

95.   The method of claim 93 or 94 wherein said method includes providing a recombinant Ca^{2+} receptor useful in said identifying step.

96.   Purified nucleic acid encoding a Ca^{2+} receptor.

97.   The compounds NPS 459, NPS 467, NPS 544 and NPS 568 and pharmaceutically acceptable acid addition salts and complexes thereof.

98.   The pharmaceutical composition of claim 25 wherein said molecule is NPS 447, NPS 448, NPS 449,

NPS 456 or NPS 551.

99.   A compound of the formula:
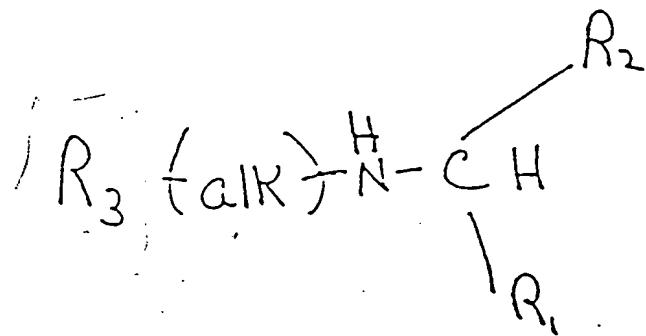
$$R_3 \text{(alk)} \overset{H}{N} - \overset{R_2}{\underset{R_1}{C}} H$$

wherein alk is straight or branched-chain alkylene of
from 1 to 6 carbon atoms; R_1 is lower alkyl of from
1 to 3 carbon atoms or lower haloalkyl of from 1 to 3
carbon atoms substituted with from 1 to 7 halogen
atoms; and R_2 and R^3 are independently selected
monocyclic or bicyclic carbocyclic aryl or cycloalkyl
groups, having 5- or 6-membered rings optionally
substituted with 1 to 5 substituents independently
selected from lower alkyl of 1 to 3 carbon atoms,
lower haloalkyl of 1 to 3 carbon atoms substituted
with 1 to 7 halogen atoms, lower alkoxy of 1 to 3
carbon atoms, halogen, nitro, amino, alkylamino,
amido, lower alkylamido of 1 to 3 carbon atoms, cyano,
hydroxy, acyl of 2 to 4 carbon atoms or lower
thioalkyl of 1 to 3 carbon atoms and pharmaceutically
acceptable salts and acid addition salts thereof.

-194-

100.   A compound according to claim 99 wherein alk is n-propylene.

101.   A compound according to claim 100 wherein R_1 is methyl.

102.   A compound according to claim 101 wherein R_2 and R_3 are independently selected optionally substituted phenyl groups.

103.   A isolated nucleic acid encoding an inorganic-ion receptor or a unique fragment thereof.

104.   An isolated nucleic acid as claimed in claim 103 encoding a human inorganic-ion receptor or a unique fragment thereof.

105.   An isolated nucleic acid as claimed in claim 103 encoding an inorganic-ion receptor that hybridizes to Sequence 1.

106.   An isolated nucleic acid as claimed in claim 103 encoding an inorganic-ion receptor that hybridizes to BoPCaR 1 or a unique fragment thereof.

107.   An isolated nucleic acid claimed in claim 103 encoding a calcium receptor or a unique fragment thereof.

SUBSTITUTE SHEET

108. An isolated nucleic acid as claimed in
claim 107 encoding a calcium receptor that is
expressed in tissues or cells selected from the group
consisting of:
      parathyroid, vascular, kidney, epidermis,
thyroid, osteoclast, intestine, mammary, trophoblast
platelet, gastrin secreting, glucagon secreting,
cardiac, and brain, and unique fragments thereof.

109. An isolated nucleic acid as claimed in
claim 103, wherein the nucleic acid is a fragment
encoding the extracellular binding domain but
substantially free of transmembrane and intracellular
domain portions.

110. A isolated nucleic acid as claimed in
claim 103, wherein the nucleic acid is a fragment
encoding the intracellular domain but substantially
free of transmembrane and extracellular binding
domains.

111. An isolated nucleic acid as claimed in
claim 109, wherein the fragment encoding the
extracellular binding domain is transcriptually
coupled to nucleic acid encoding transmembrane and
intracellular coding domains of a noninorganic-ion
receptor.

112. An isolated nucleic acid as claimed in
claim 103, comprising BoPCaR 1 or a unique fragment
thereof.

SUBSTITUTE SHEET

113. The isolated nucleic acid of any one of claims 103, 104, 105, and 106 under the transcriptional control of an exogenous promoter.

114. A recombinant cell expressing the nucleic acid of any one of claims 103, 104, 105, 106 and 107.

115. An isolated inorganic-ion receptor, or a unique fragment thereof.

116. An isolated inorganic-ion receptor as claimed in claim 115 that is a human inorganic-ion receptor, or a unique fragment thereof.

117. An isolated inorganic-ion receptor as claimed in claim 115 or 116 wherein the receptor is a calcium receptor.

118. An isolated inorganic-ion receptor as claimed in claim 115 that is a calcium receptor expressed in tissues or cells selected from the group consisting of:
        parathyroid, vascular, kidney, epidermis, thyroid, osteoclast, intestine, mammary, trophoblast platelet, gastrin secreting, glucagon secreting, cardiac, and brain, and unique fragments thereof.

119. An isolated inorganic-ion receptor as claimed in claim 115 that is the expression product of BoPCar 1, or a unique fragment thereof.

SUBSTITUTE SHEET

120. An isolated inorganic-ion receptor as claimed in claim 115 that is a human parathyroid calcium receptor or unique fragment thereof.

121. An isolated inorganic-ion receptor as claimed in claim 13 that is an extracellular, ion-bonding fragment substantially free of intracellular and transmembrane domains.

122. An isolated inorganic-ion receptor as claimed in claim 111 that is a calcium-binding fragment.

123. Purified antibodies to an inorganic-ion receptor.

124. Purified antibodies as claimed in claim 123 to an extracellular domain of an inorganic-ion receptor.

125. Purified antibodies as claimed in claim 123 to a calcium receptor.

126. Purified antibodies as claimed in claim 125 to a calcium receptor expressed in tissue or cells selected from the group consisting of:
    parathyroid, vascular, kidney, epidermis, thyroid, osteoclast, intestine, mammary, trophoblast platelet, gastrin secreting, glucagon secreting, cardiac, and brain, and unique fragments thereof.

SUBSTITUTE SHEET

127.   Purified antibodies as claimed in claim
124 to a calcium receptor.

128.   Purified antibodies as claimed in claim
127 to an allosteric site of a calcium receptor.

129.   Purified antibodies as claimed in claims
124, 125, 126 or 127 coupled to a toxin.

130.   An inorganic-ion receptor binding agent
coupled to a toxin.

131.   An agent as claimed in claim 131 wherein
the agent selectively binds a calcium receptor.

132.   A transgenic, nonhuman mammal containing a
transgene encoding an inorganic-ion receptor or a
unique fragment thereof.

133.   A transgenic mammal as claimed in claim
132 wherein the transgene encodes a calcium receptor
or a unique fragment thereof.

134.   A transgenic, nonhuman mammal containing a
transgene that alters the expression of an
inorganic-ion receptor.

135.   A transgenic mammal as claimed in claim
134 wherein the transgene inactivates the expression
of the inorganic-ion receptor.

136. A transgenic mammal as claimed in claim 134 wherein the transgene up-regulates or down-regulates the expression of the inorganic-ion receptor.

137. A transgenic mammal as claimed in claims 134, 135 or 136 wherein the transgene alters the expression of a calcium receptor.

138. A method for identifying nucleic acid encoding an inorganic-ion receptor comprising
        contacting a library of nucleic acid with a probe selected from the group consisting of:
           (a)   BoPCaR 1
           (b)   a unique fragment of BoPCaR 1
           (c)   the human homolog of BoPCaR 1
           (d)   a set of degenerate probes of any of
(a), (b) or (c) and identifying sequences within the library that hybridize to the probe.

139. A method as claimed in claim 138 further comprising
        isolating the nucleic acid encoding the inorganic-ion receptor.

140. A method as claimed in claim 138 further comprising
        identifying a tissue in which the inorganic-ion receptor is expressed.

SUBSTITUTE SHEET

141.   A method for modulating the expression of
an inorganic-ion receptor comprising
        contacting a cell with an agent that binds
to a cellular component selected from the group
consisting of:
        a promoter;
        a regulatory agent acting on a promoter;
        an agent capable of binding to the
receptor; and
        a nucleic acid encoding the receptor or a
unique fragment thereof.

142.   A method as claimed in claim 141 wherein
the cell is contacted with an antisense nucleic acid.

143.   A method as claimed in claim 141 wherein
the cell is contacted with a DNA constructed and
arranged to combine through homologous recombination
with an endogenous gene encoding the receptor and to
inactivate expression of the receptor in the cell.

144.   A method for killing a cell expressing an
inorganic-ion receptor comprising
        contacting the cell with an agent capable
of binding to the inorganic-ion receptor, wherein the
agent is conjugated to a toxin.

145.   A method for identifying an agent that is
an agonist or an antagonist of an inorganic-ion
receptor, comprising

-201-

contacting a cell containing a recombinant
nucleic acid encoding an inorganic-ion receptor with
the agent and detecting a change in the cell.

146. A method for identifying an agent that
binds to an inorganic-ion receptor comprising an assay
employing an isolated inorganic-ion receptor.

FIG. 1a.

NPS 019

BUDMUNCHIAMINE A (+3)

NPS 383 (+8 EC$_{50}$=150μM)

NPS 381 (+10 EC$_{50}$=100μM)

SPERMIDINE +3

1,12-DIAMINODODECANE -2
(DADD)

TETRAETHYLENEPENTAMINE (+5 EC$_{50}$=2500μM)

TRIETHYLENETETRAMINE (+4 EC$_{50}$=8000μM)

PENTAETHYLENEHEXAMINE (+6 EC$_{50}$=500μM)

NPS 021
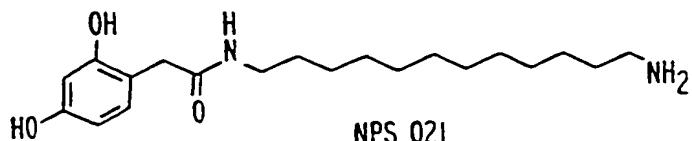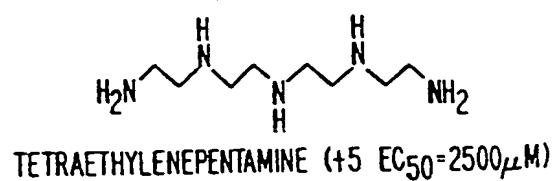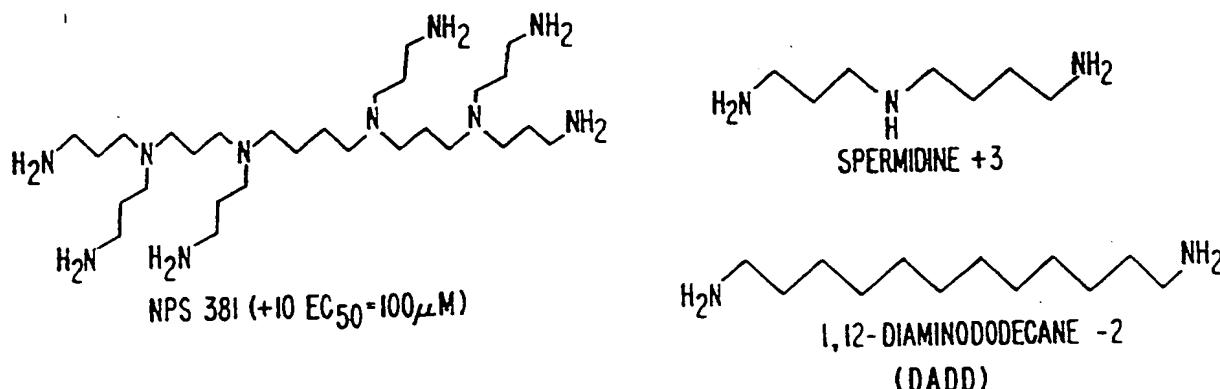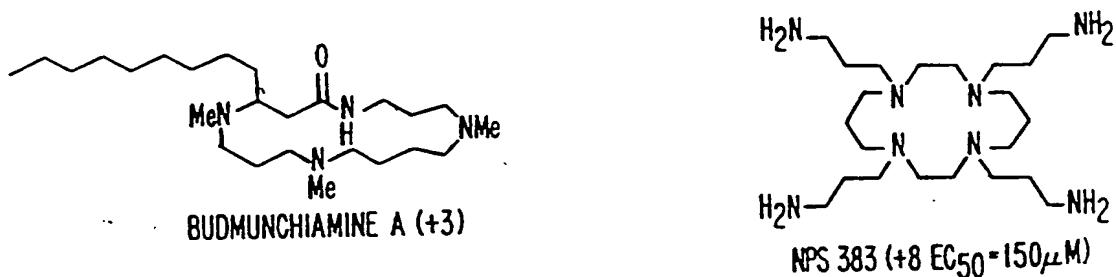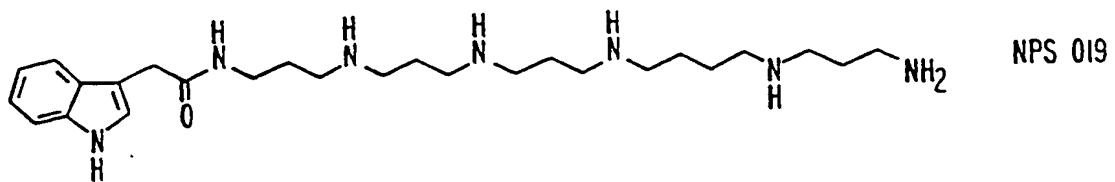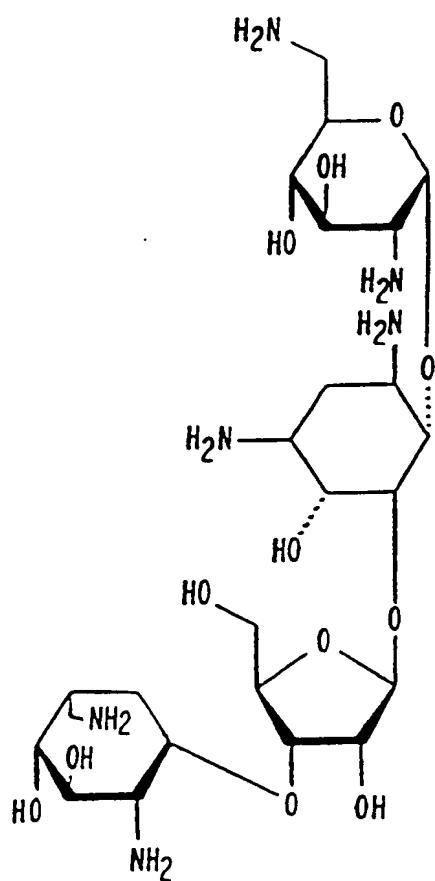
SUBSTITUTE SHEET

Neomycin B (+6)
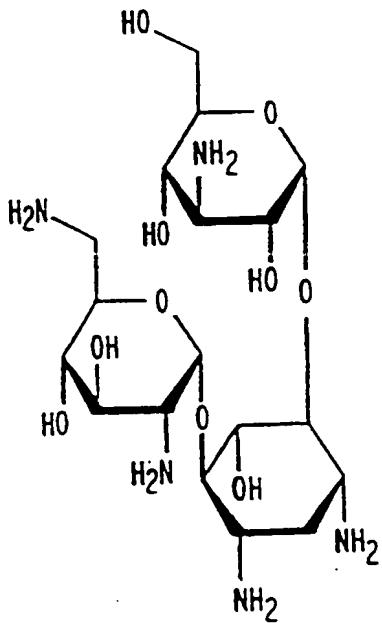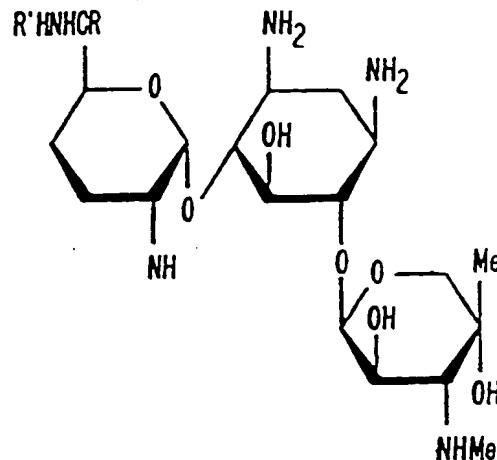
Gentamicin (Complex +5)
C1 R = R' = Me
C2 R = Me; R' = H
C1a R = R' = H

Bekanamycin (+5)
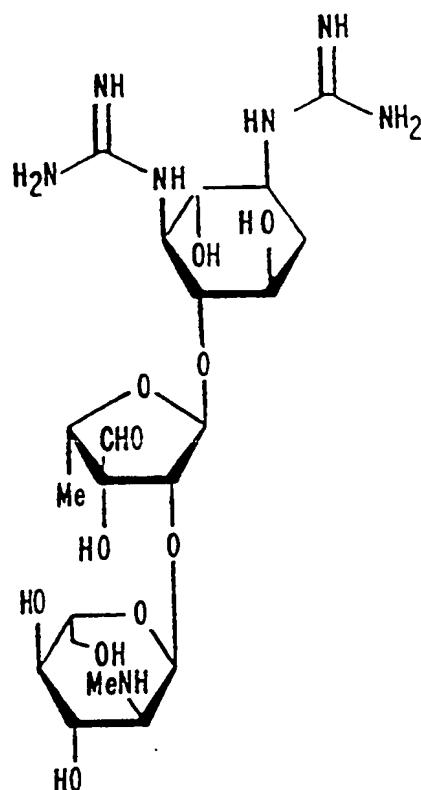
Streptomycin (+3)

FIG. 1b.

SUBSTITUTE SHEET
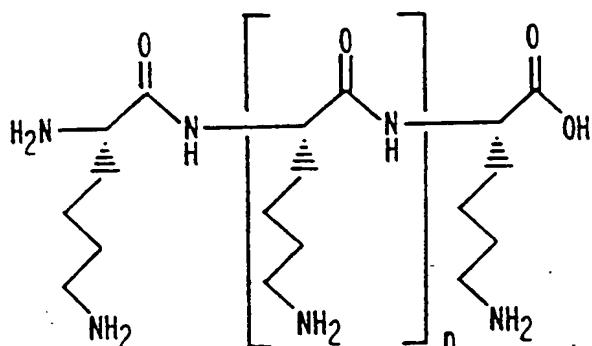
poly-Lys (27 kD) n=210 +210
poly-Lys (14kD) n=110 +110
poly-Lys (38kD) n=55 +55
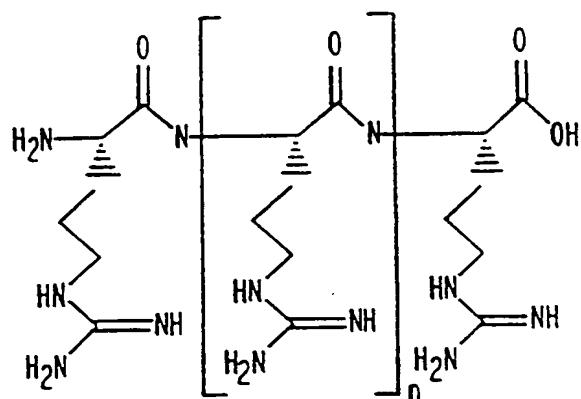
poly-Arg (100kD) n=640 +640
poly-Arg (40kD) n=256 +256

PROTAMINE +21

H N-Pro-Arg-Arg-Arg-Arg-Ser-Ser-Ser-Arg-Pro-Val-Arg-Arg-Arg-Arg-
Pro-Arg-Val-Ser-Arg-Arg-Arg-Arg-Arg-Arg-Gly-Gly-Arg-Arg-Arg-Arg-OH

FIG. Ic.

VERAPAMIL R=H +1
0-600 R=OMe +1

NIFEDIPINE +1

NITRENDIPINE +1

BAY K 8644 +1

202-791 +1

DILTIAZEM +1
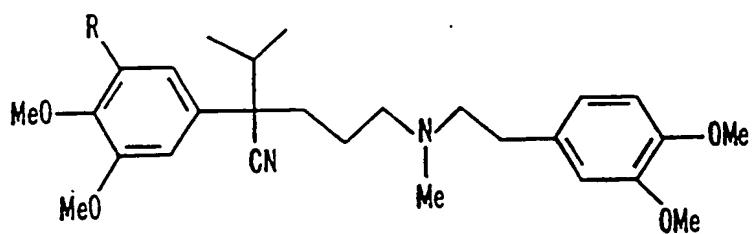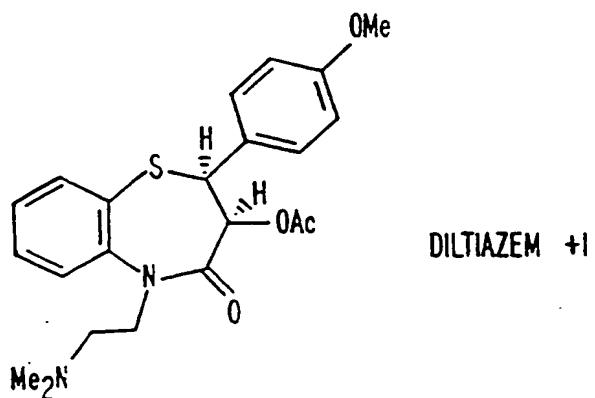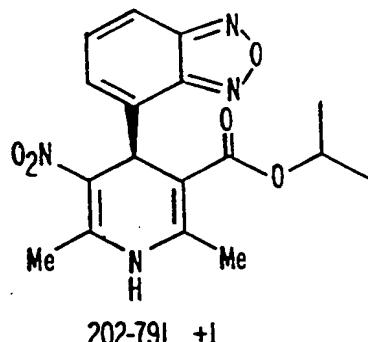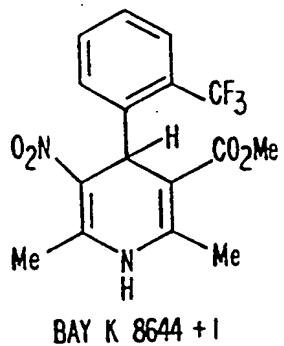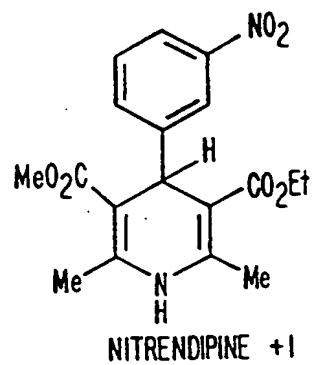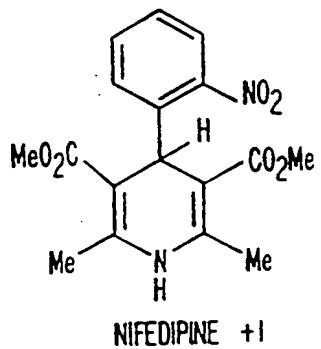
FIG. Id.
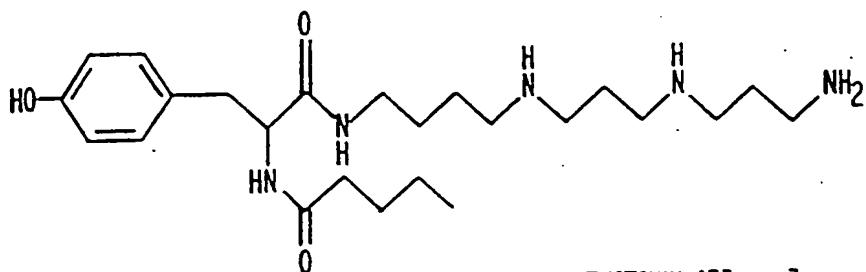
PHILANTHOTOXIN 433   +3
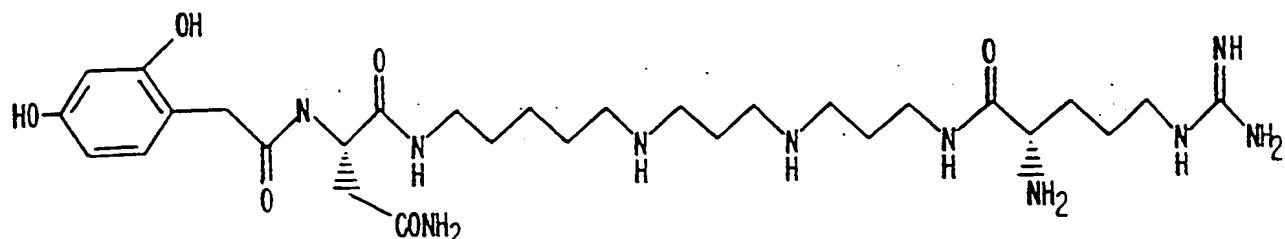
ARGIOTOXIN 636   +4
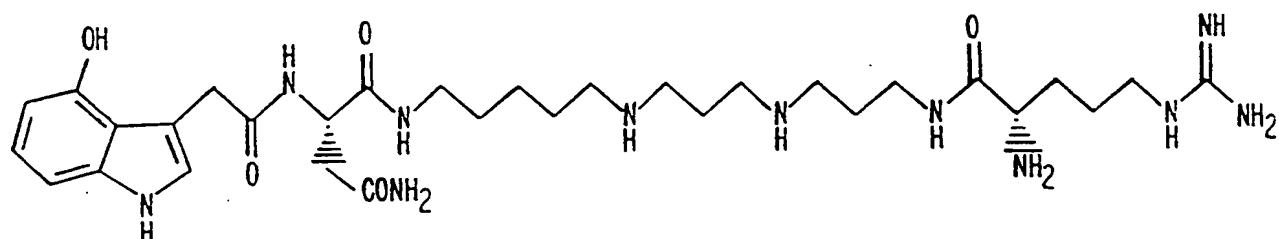
ARGIOTOXIN 659   +4

NPS 384

TMB-8   +1

FIG. Ie.

SUBSTITUTE SHEET

AGA 489

AGA 505

SPERMINE   141.148

HEXACYCLEN   20.9

NPS 382   55.0
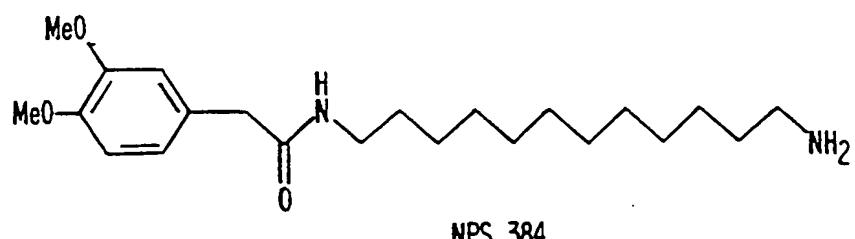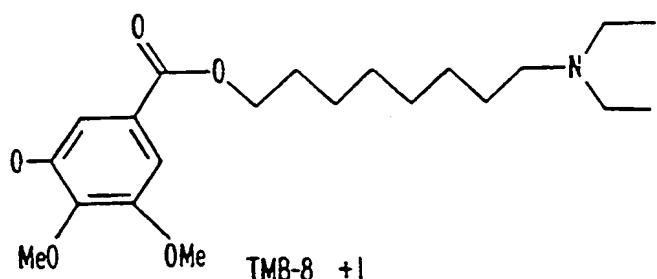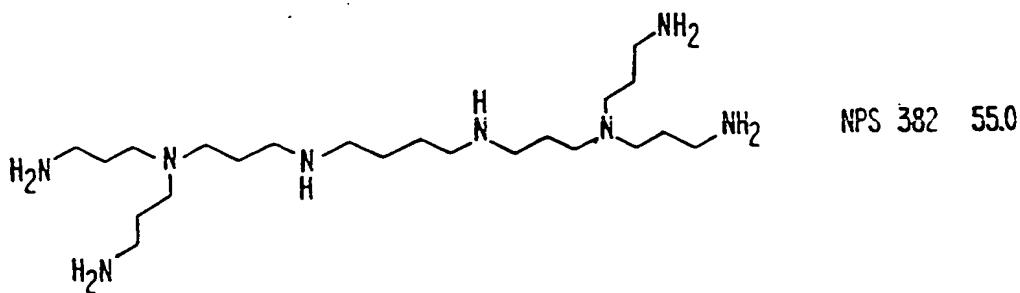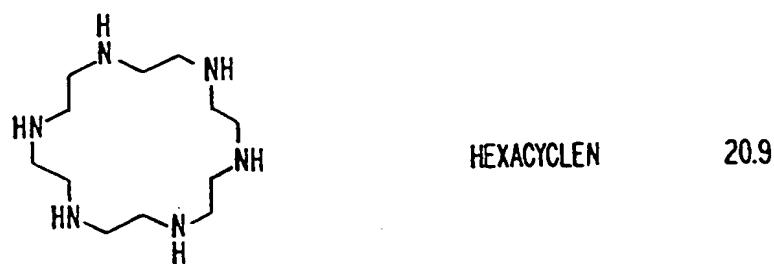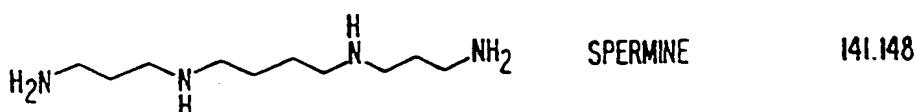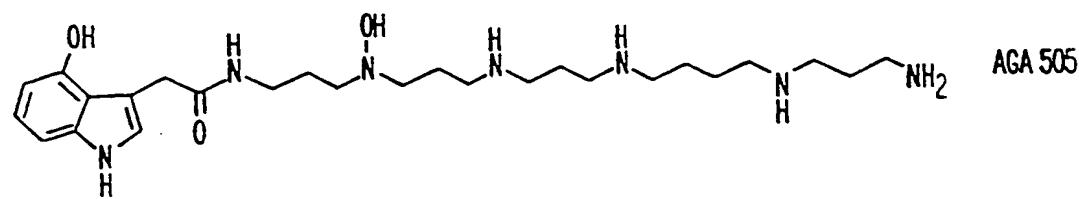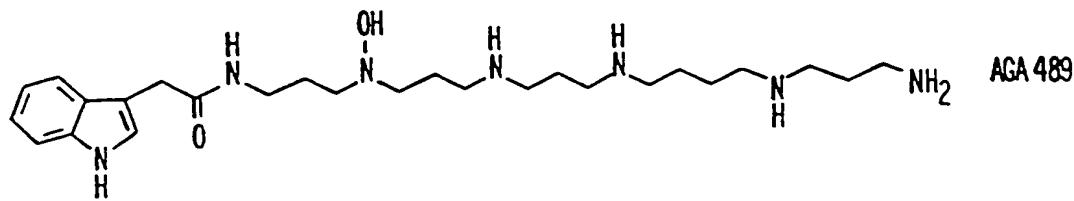
FIG. 1f.

FIG. 2.
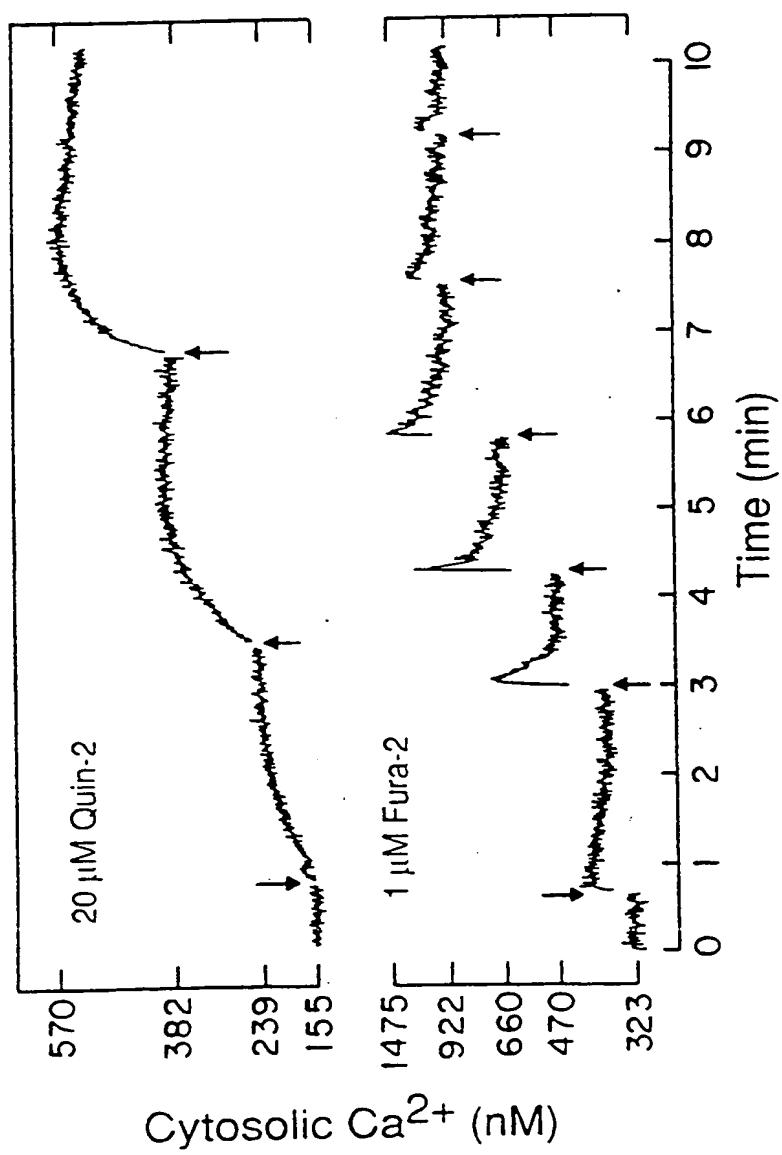
FIG. 3a.
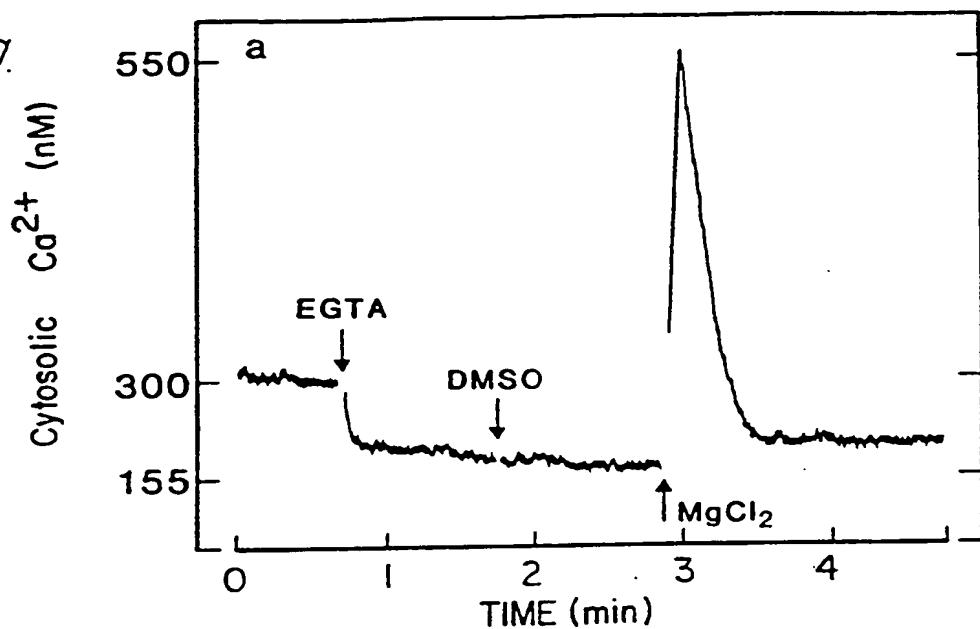
FIG. 3b.

FIG. 3c.

FIG. 4a.
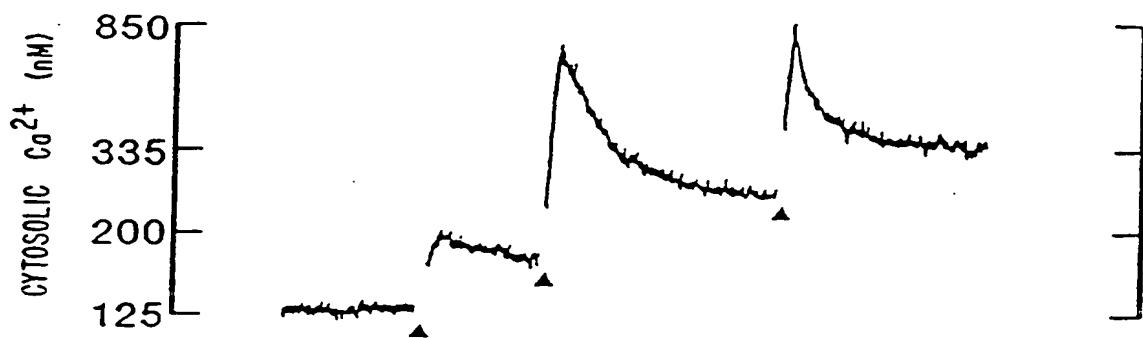
FIG. 4b.
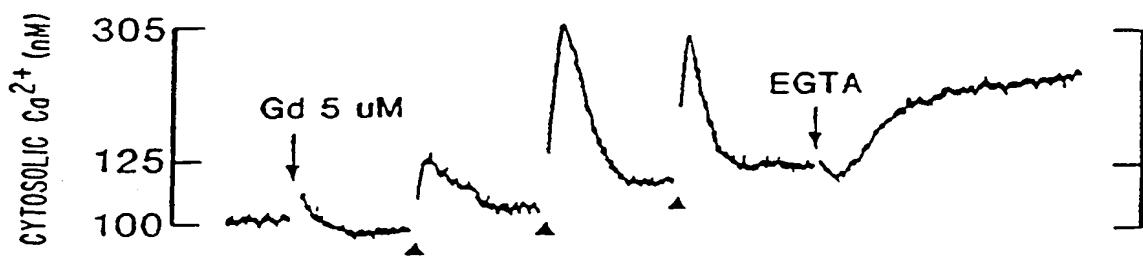
FIG. 4c.
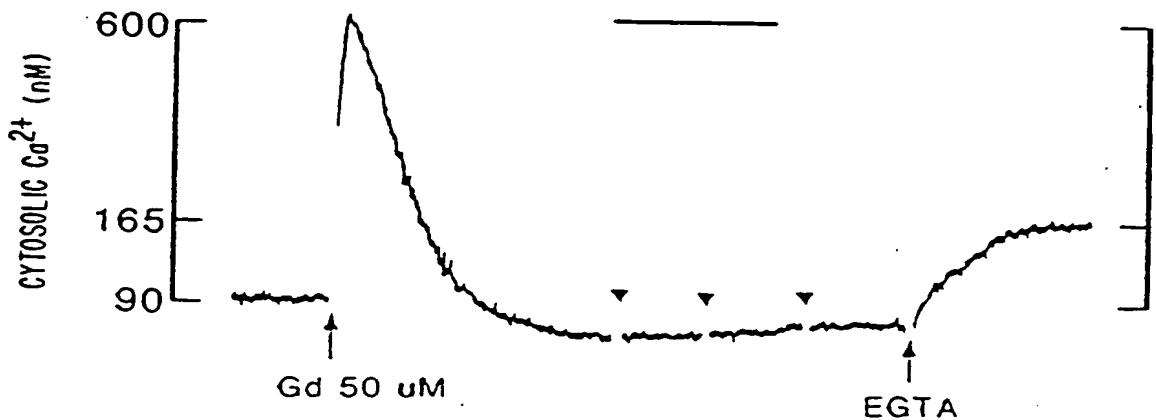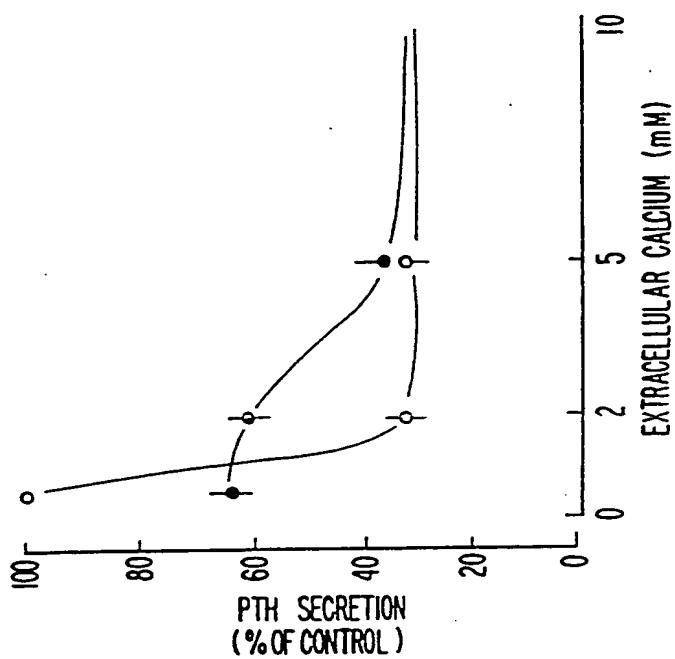
SUBSTITUTE SHEET

FIG. 5c.

PTH SECRETION
(% OF CONTROL)

EXTRACELLULAR CALCIUM (mM)

FIG. 5b.

IP₃ (% CONTROL)

EXTRACELLULAR CALCIUM (mM)

FIG. 5a.

CYTOSOLIC CA²⁺ (nM)

EXTRACELLULAR CALCIUM (mM)

FIG. 6.

FIG. 7.

FIG. 8a.

FIG. 8b.

FIG. 9a.

CONTROL ○

+ 100nM PMA ●

FIG. 9b.

CONTROL □

+ 100nM PMA ■

FIG. 9c.

CONTROL ○
+ 100nM PMA ●

FIG. 10a.

FIG. 10b.

FIG. 10c.
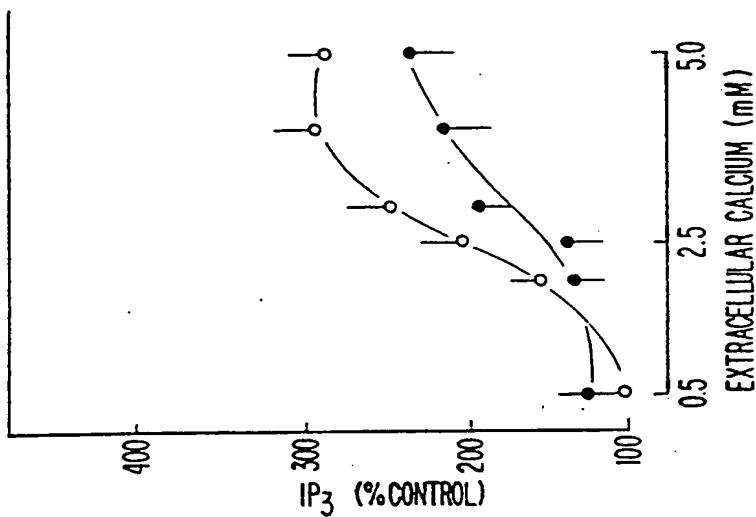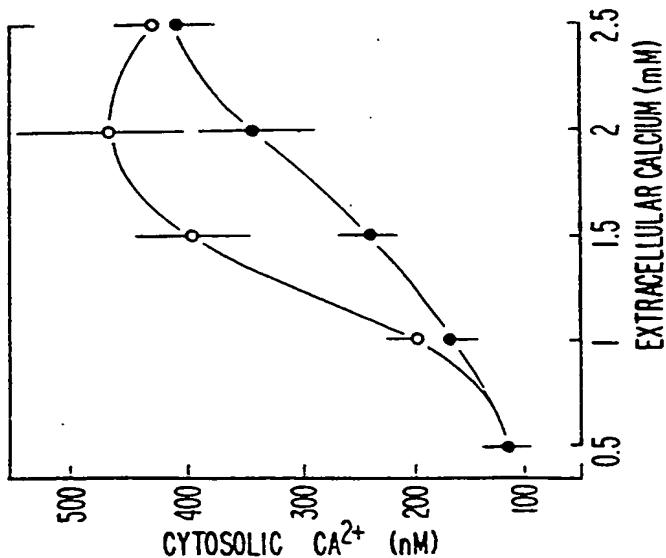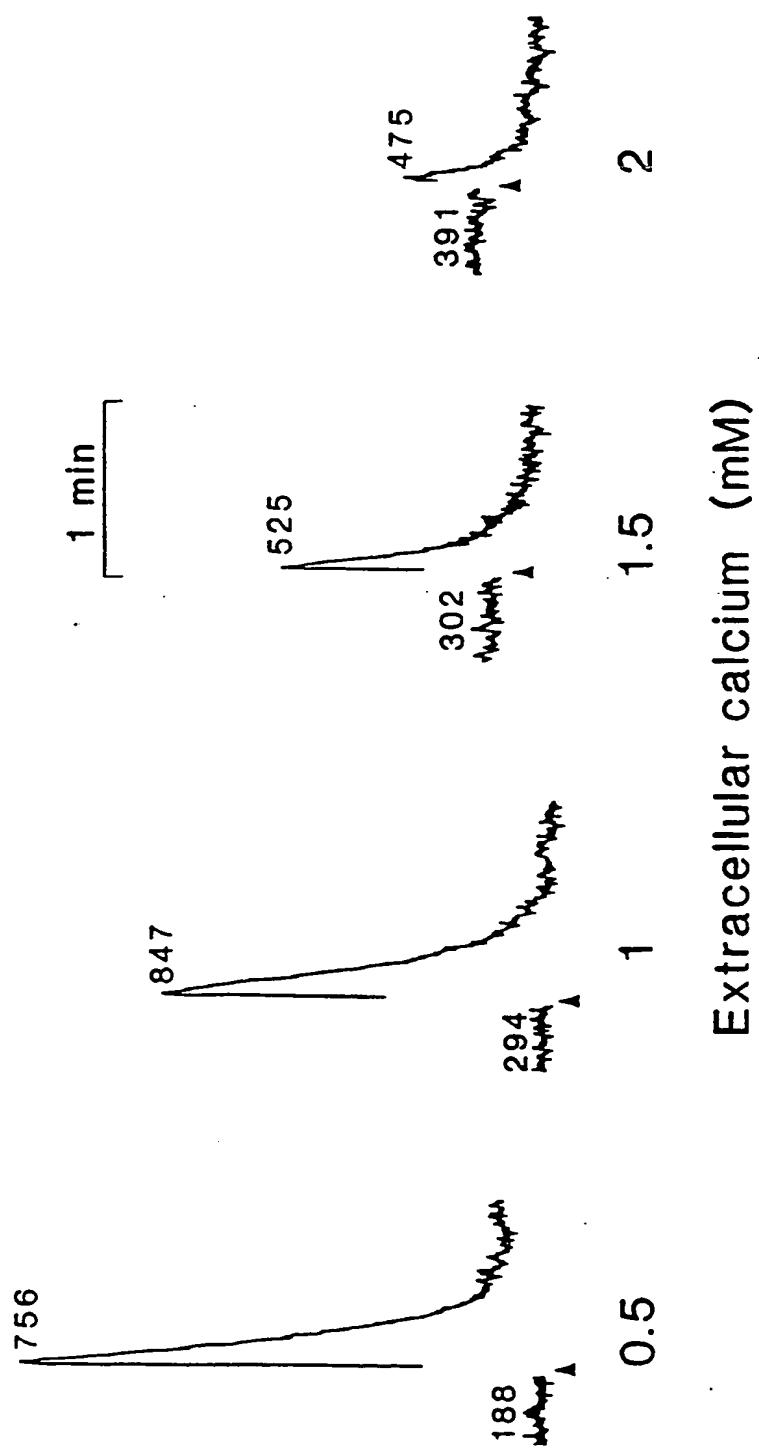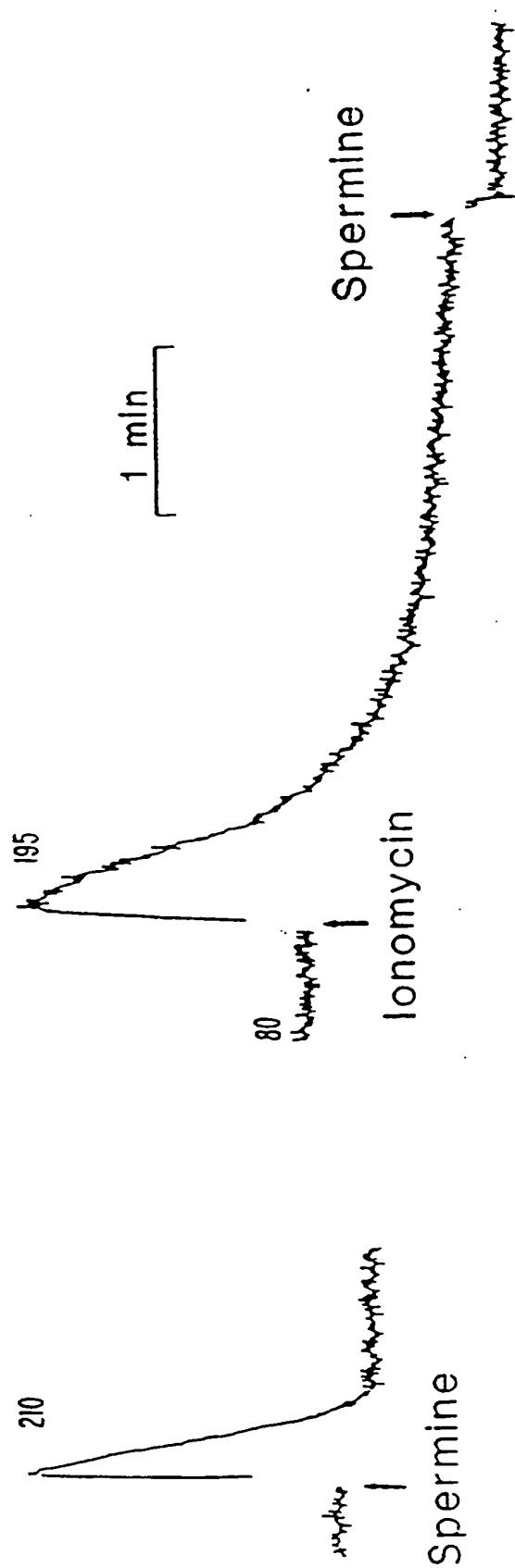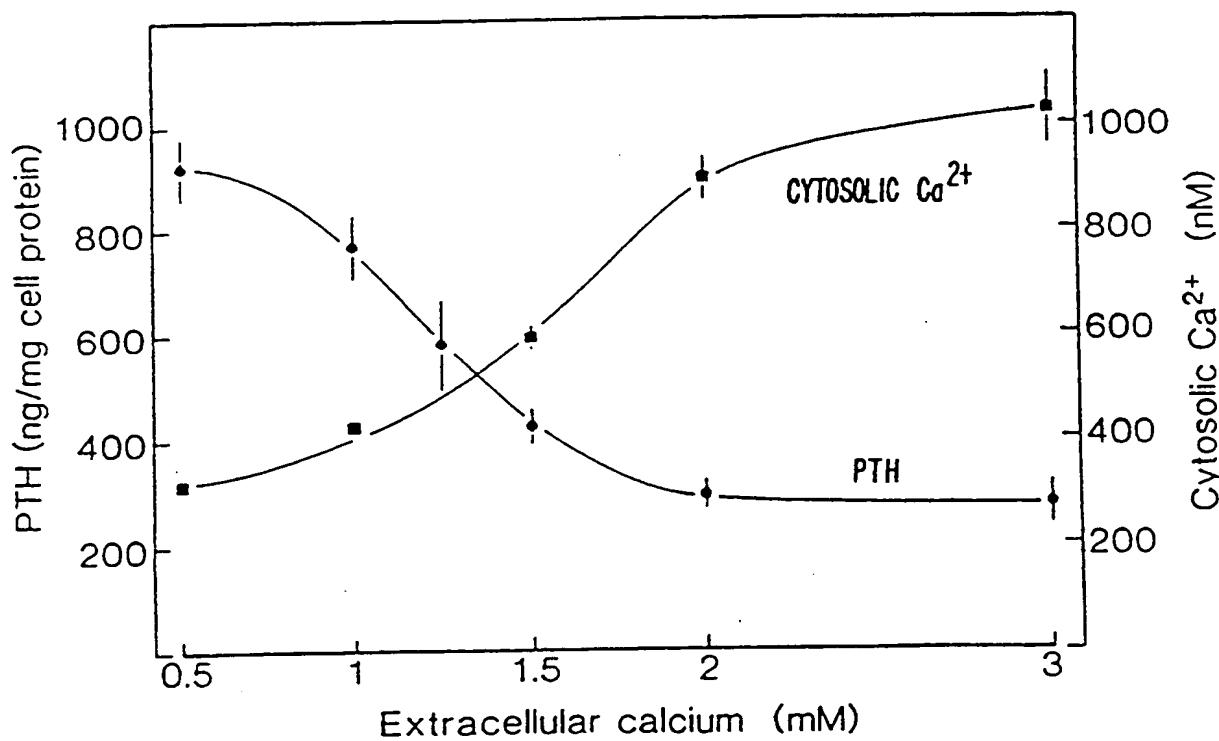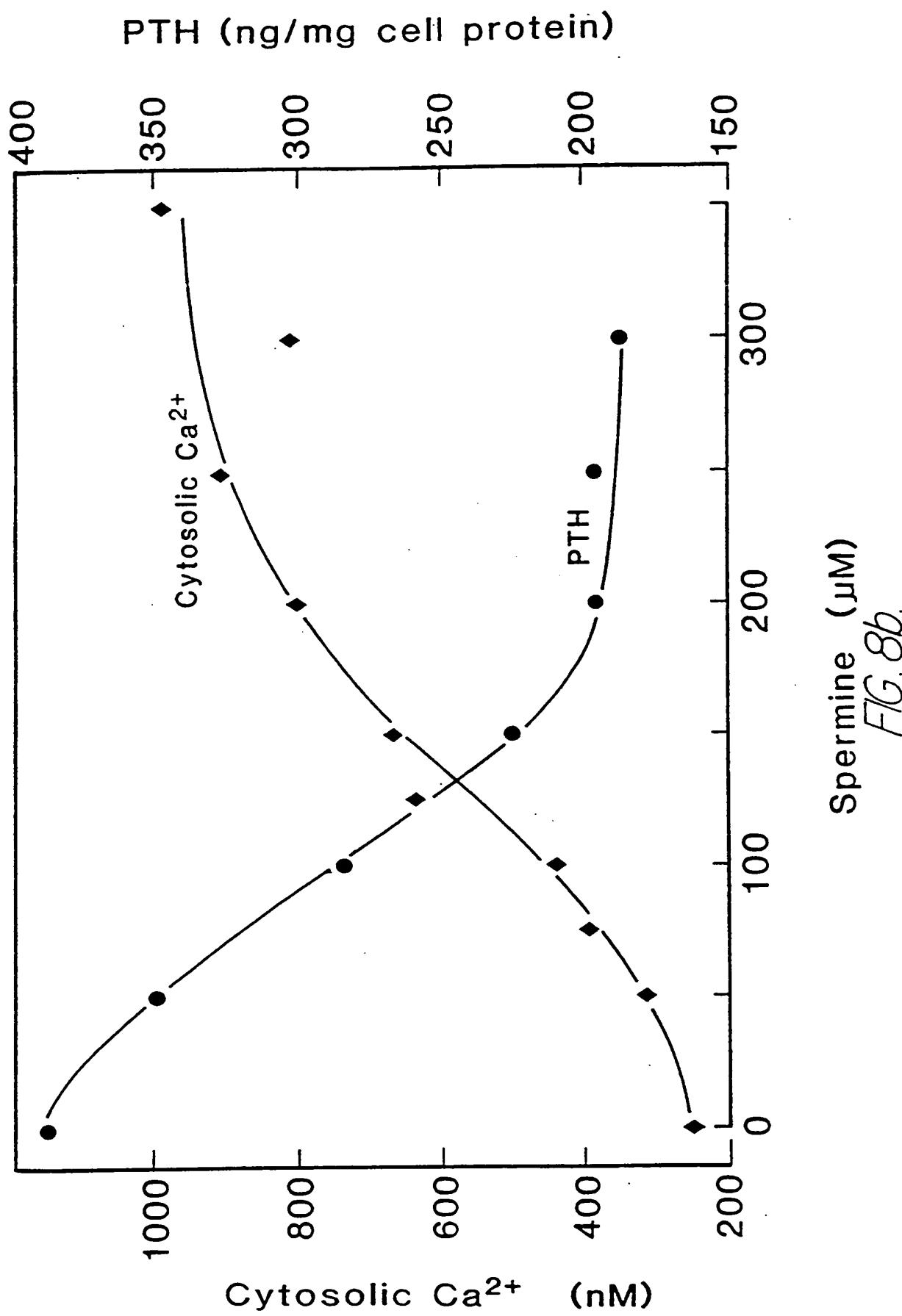
FIG. 11a.
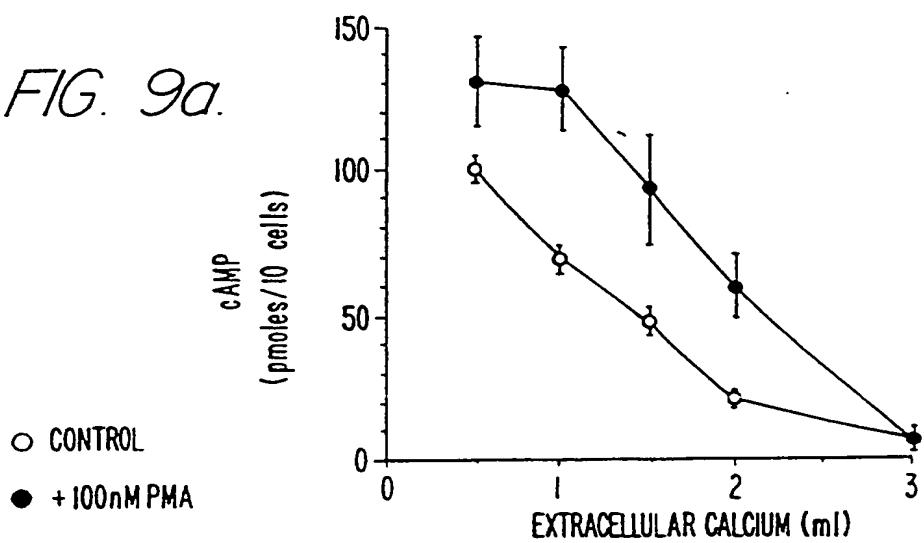
FIG. 11b.
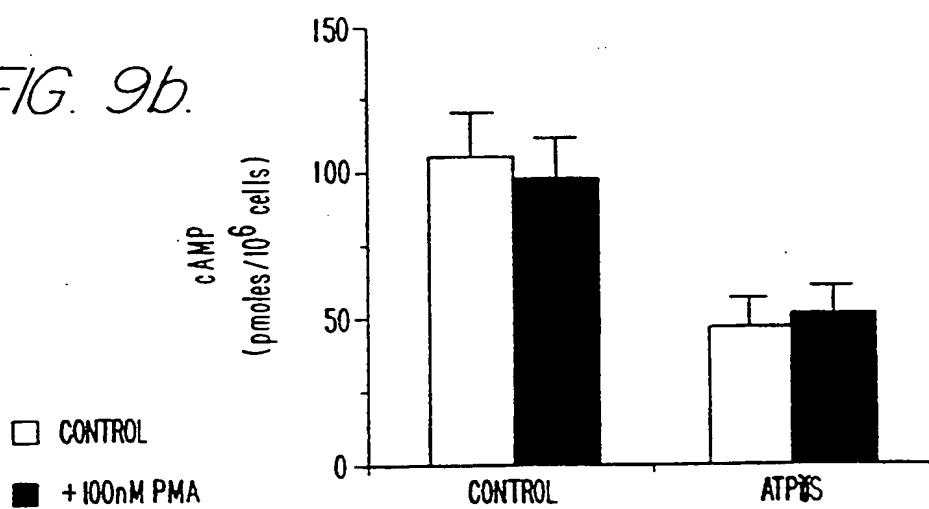
FIG. 11c.
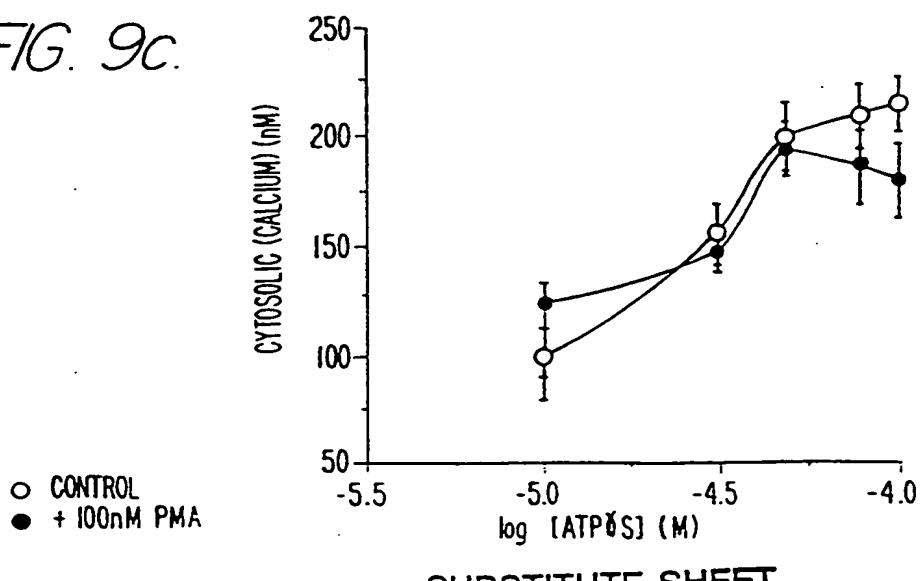
FIG. 11d.

FIG. 11e.

FIG. 11f.
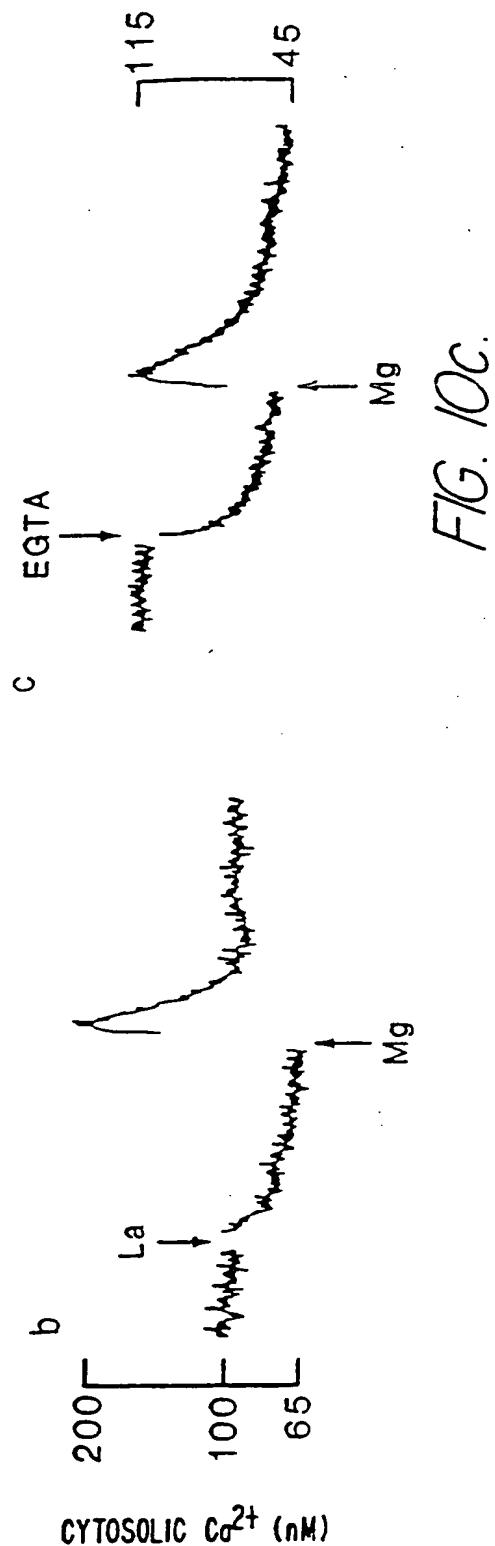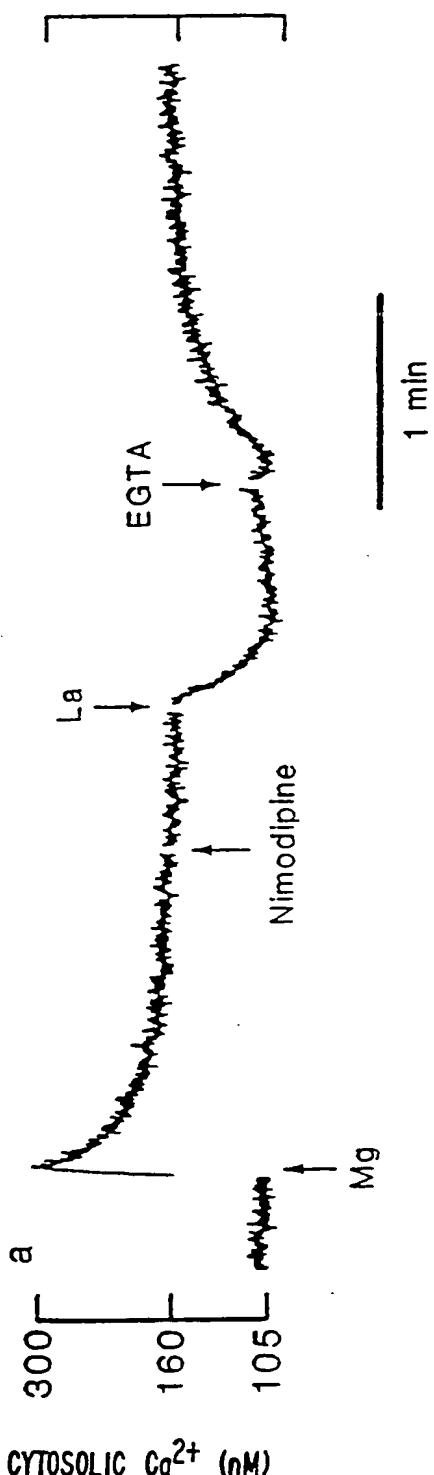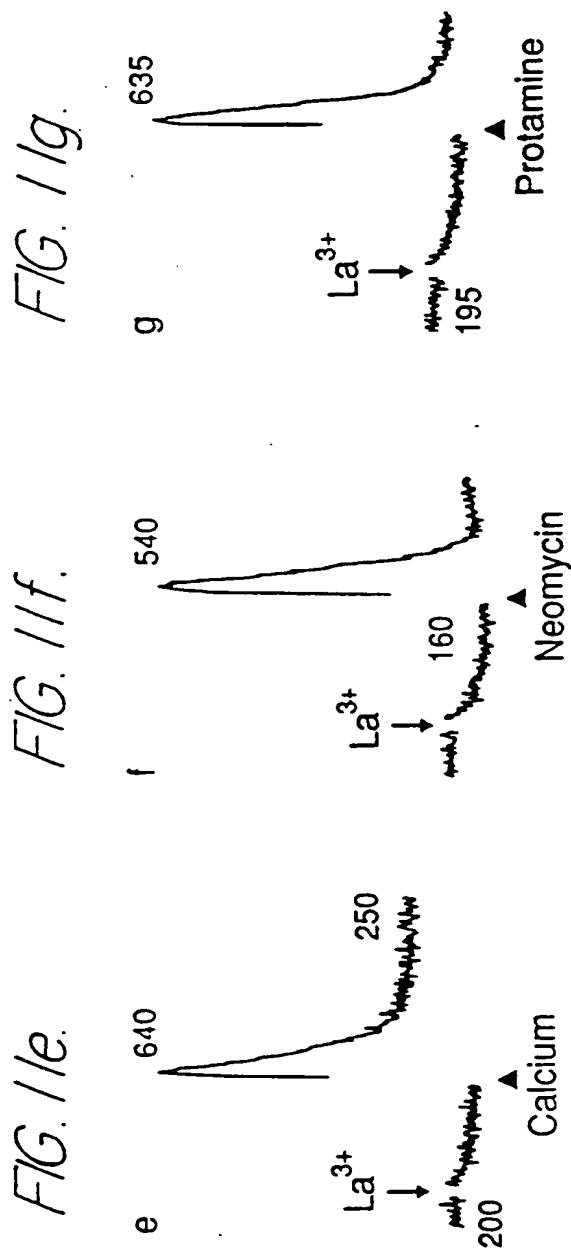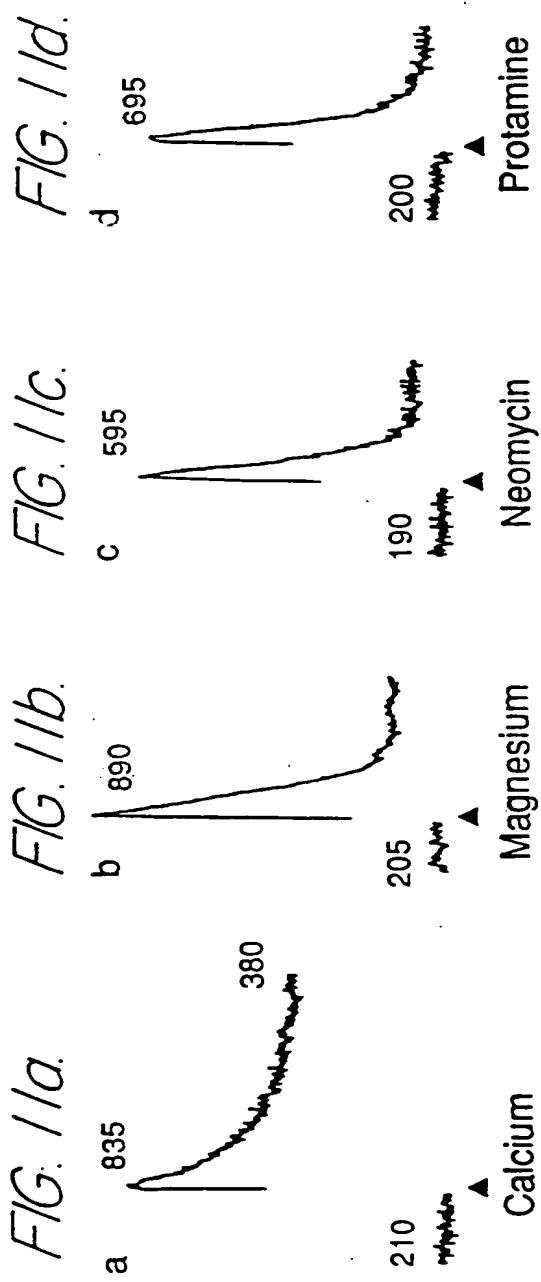
FIG. 11g.

FIG. 11i.

FIG. 11h.

FIG. 12.

FIG. 13a.

FIG. 13b.

FIG. 14.

Spermine

PMA

Spermine

ATP

ATP

FIG. 15b.

FIG. 15a.

FIG. 16b.

FIG. 16a.

Spermine (uM)

Extracellular calcium (mM)
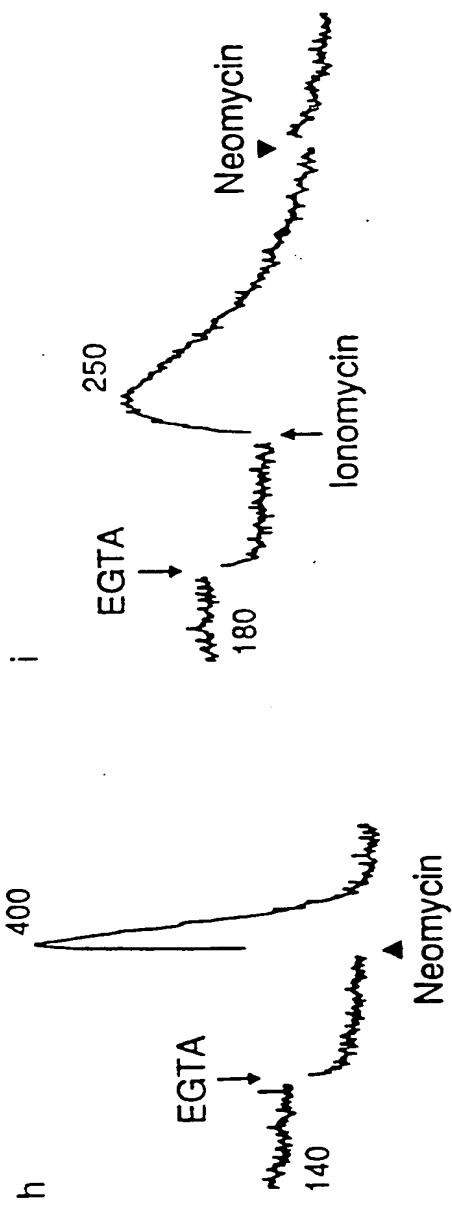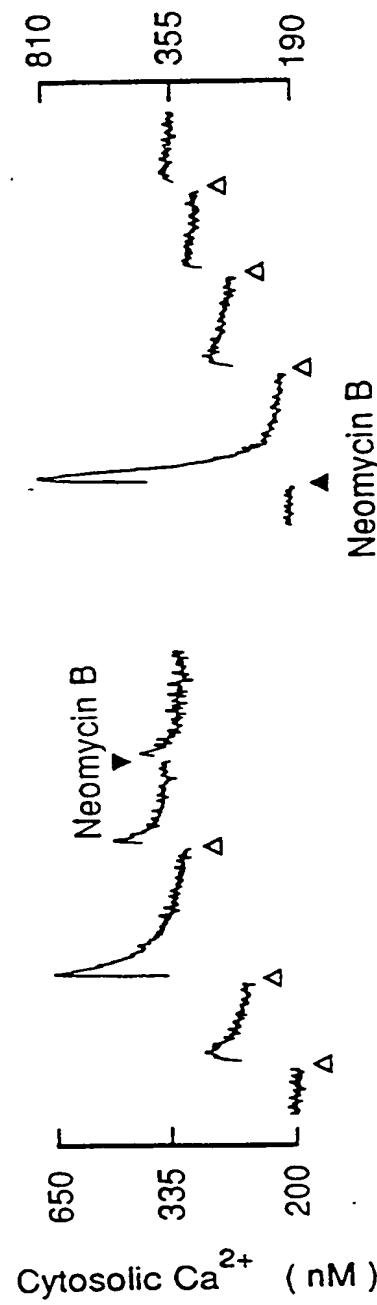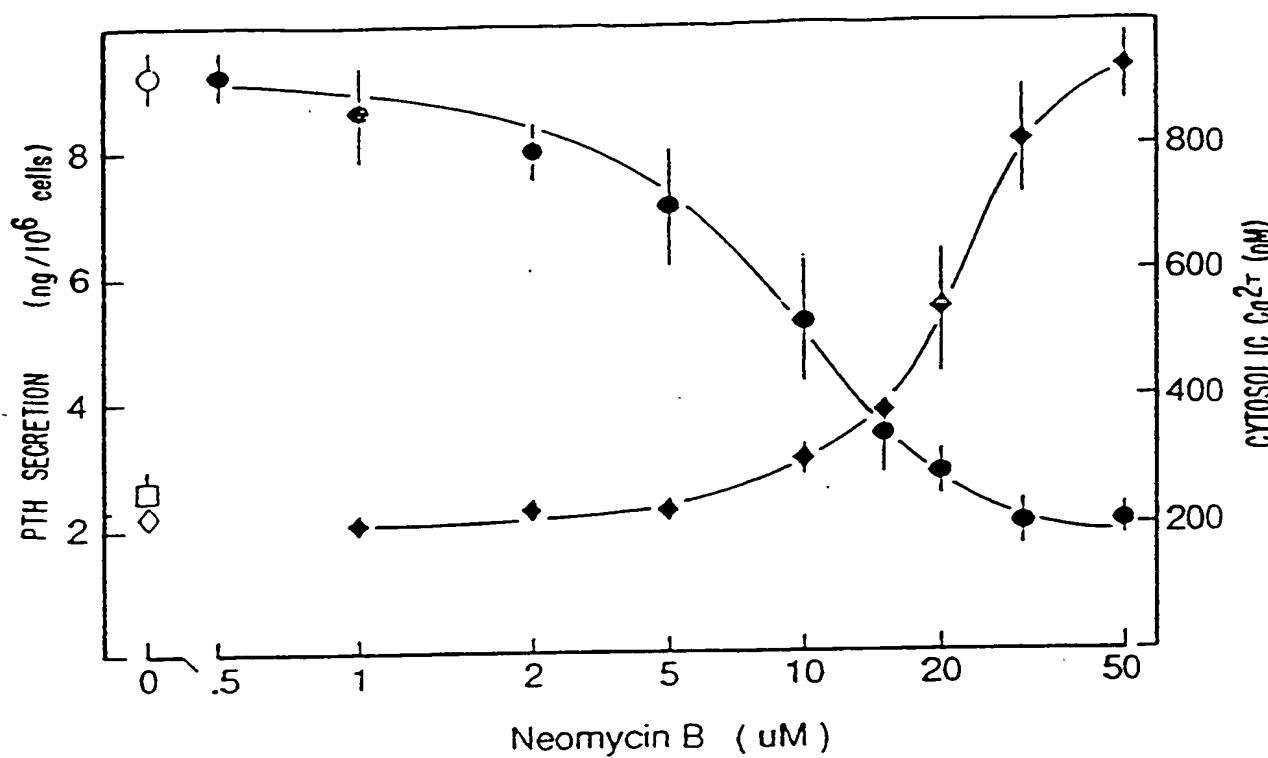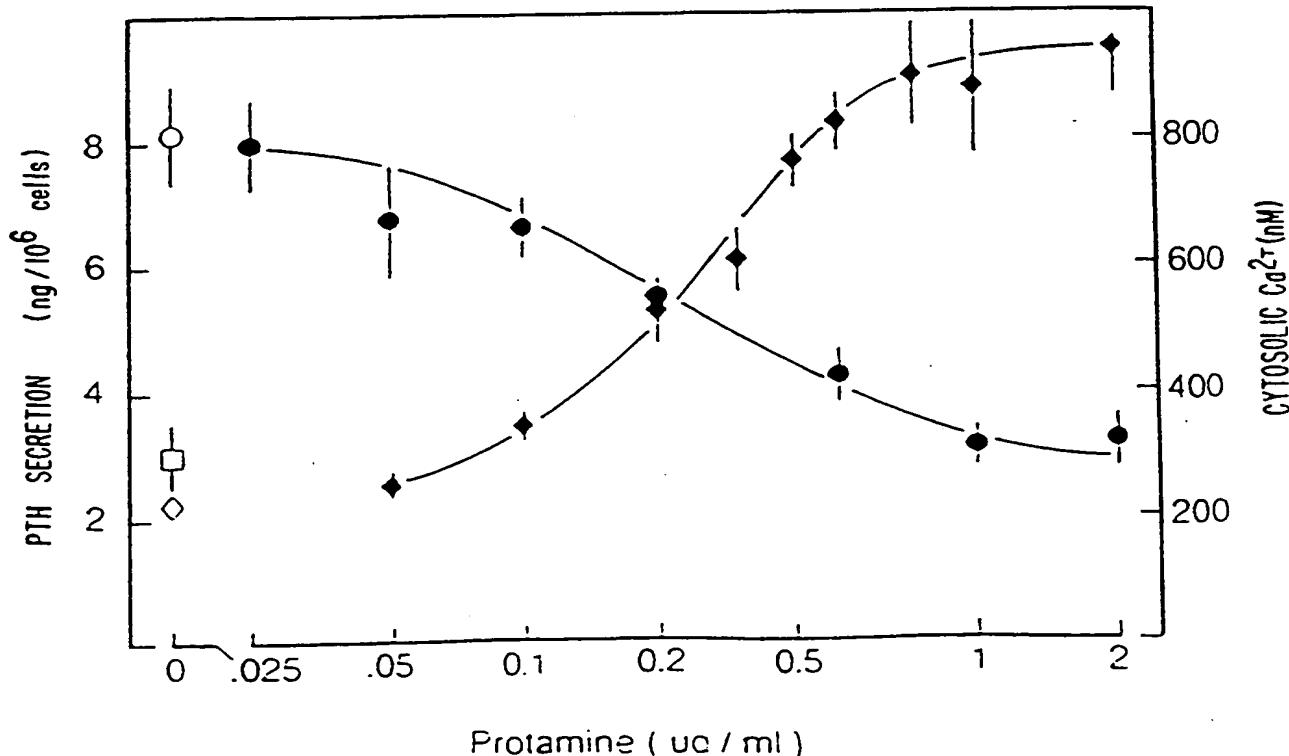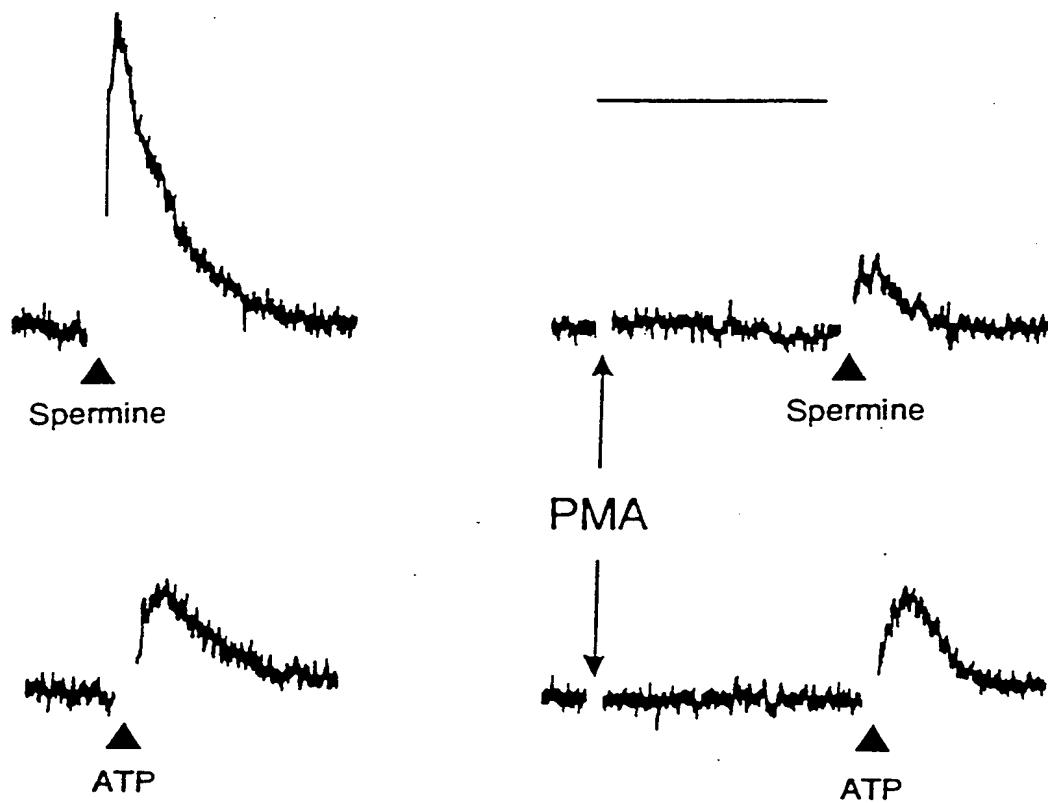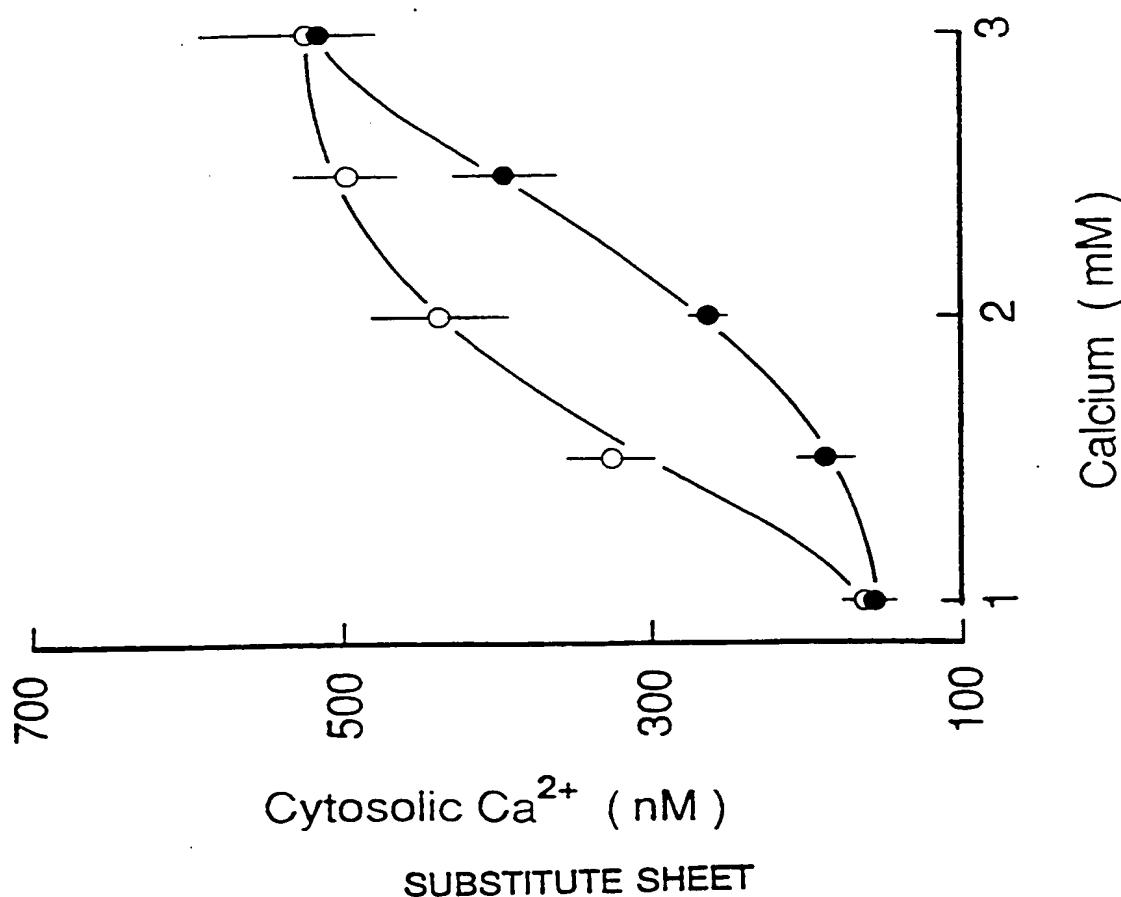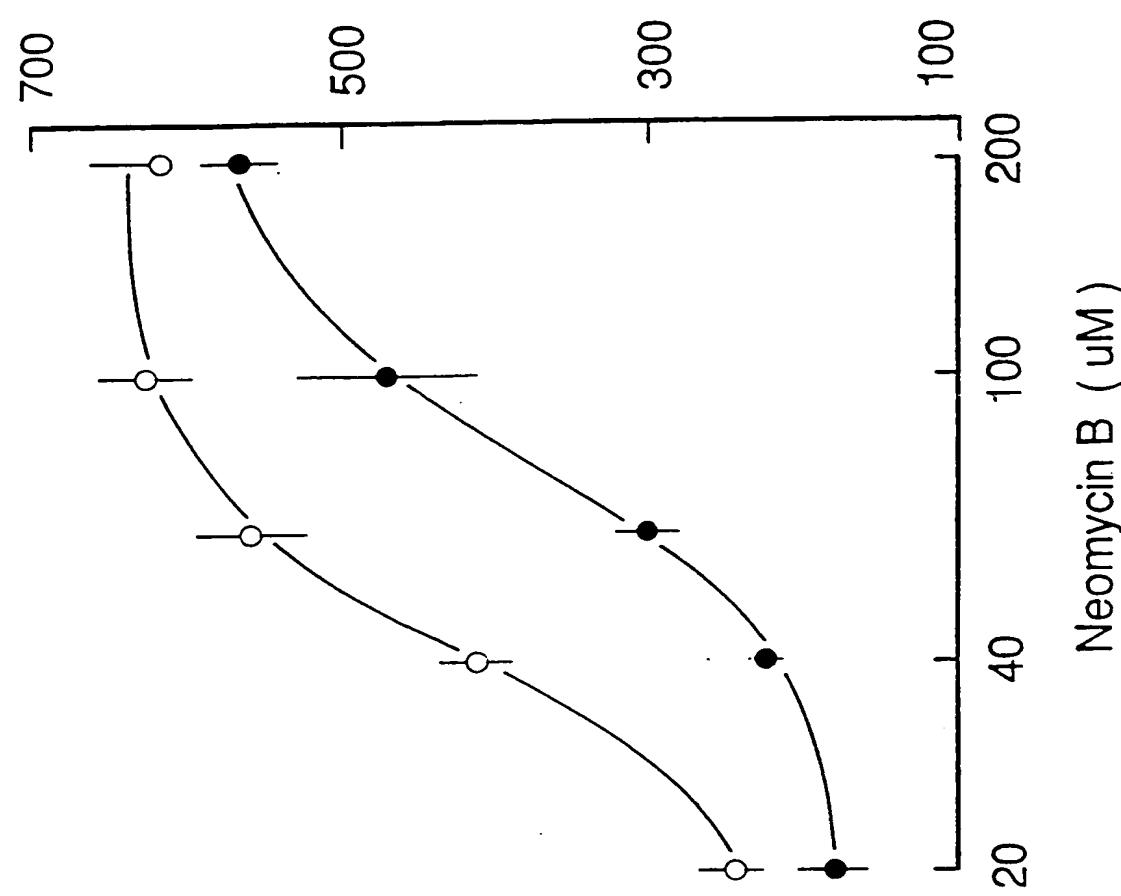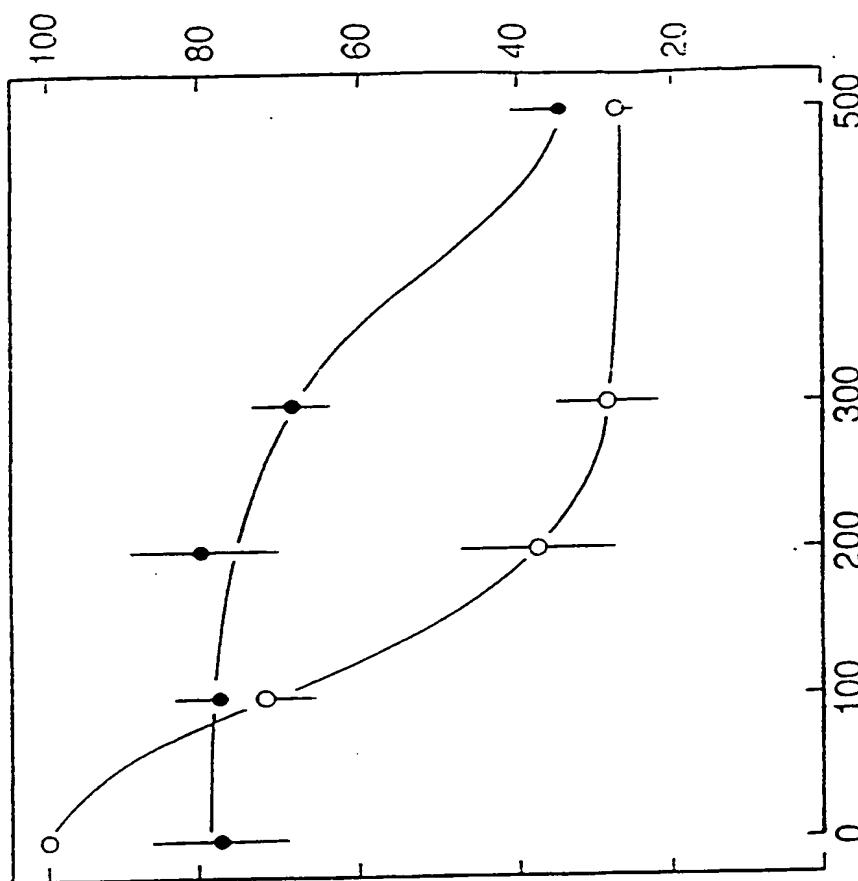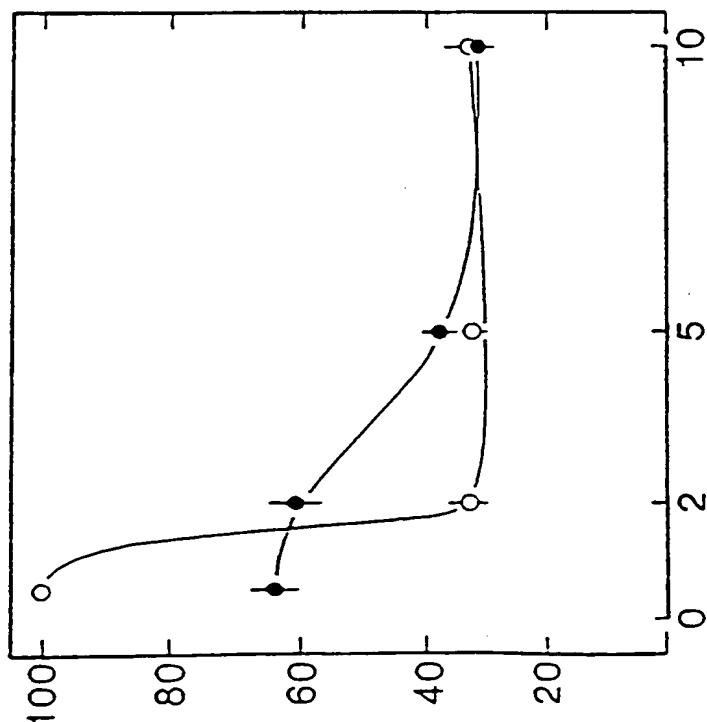
PTH Secretion
(percent of control)
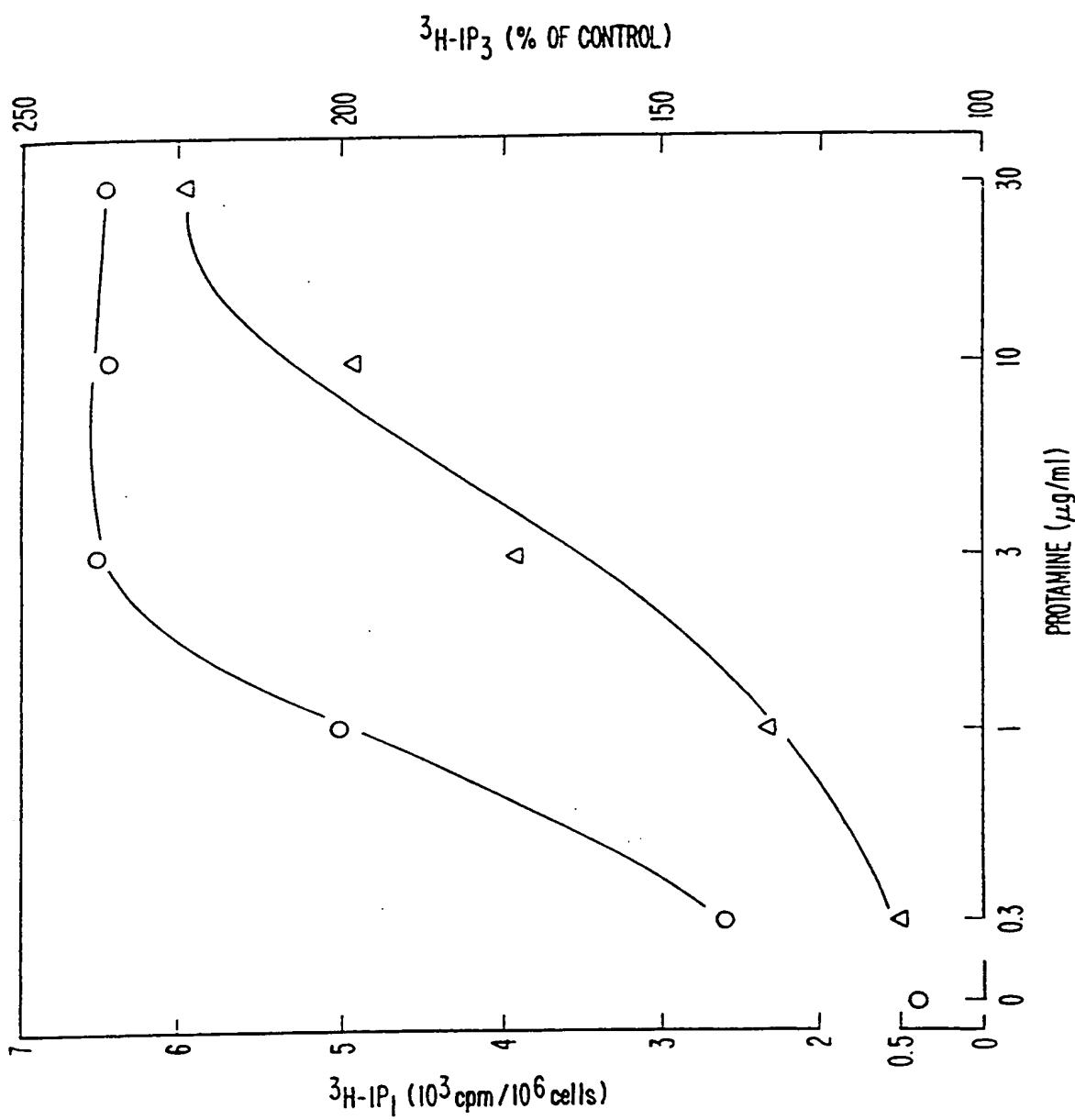
FIG. 17.

FIG. 18b.

FIG. 18a.

FIG. 19.

FIG. 20a.

1 mM Neomycin B

100 nA

2 min

FIG. 20b.

1 mM Carbachol

1 mM Neomycin B

50 nA

2 min

FIG. 21.
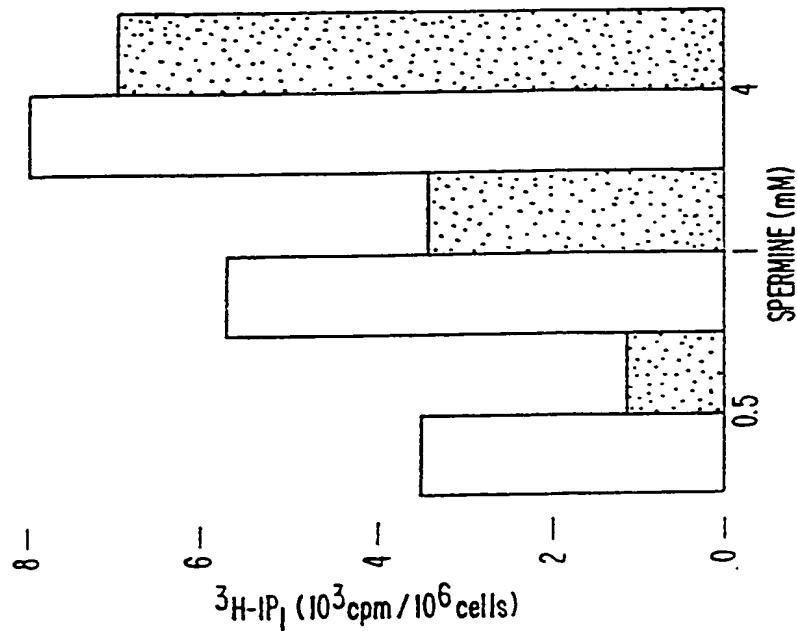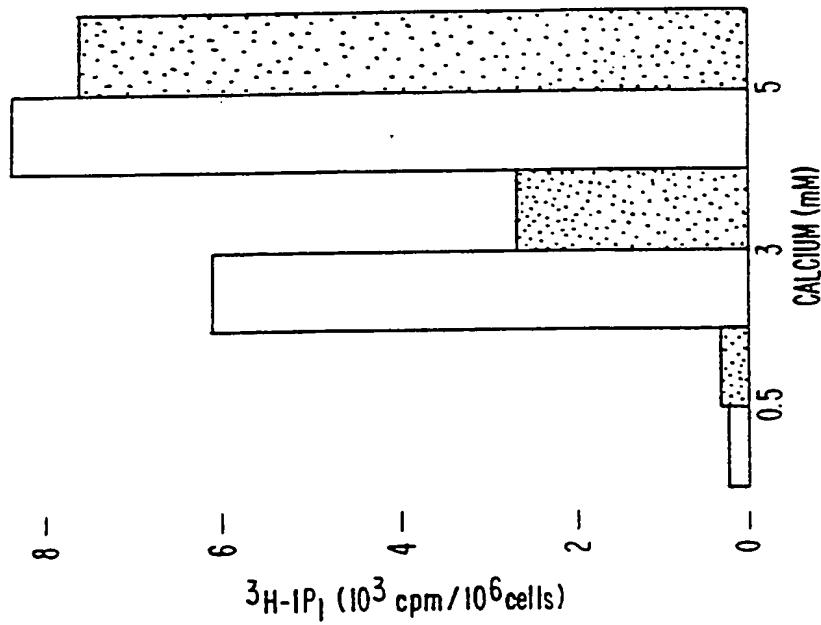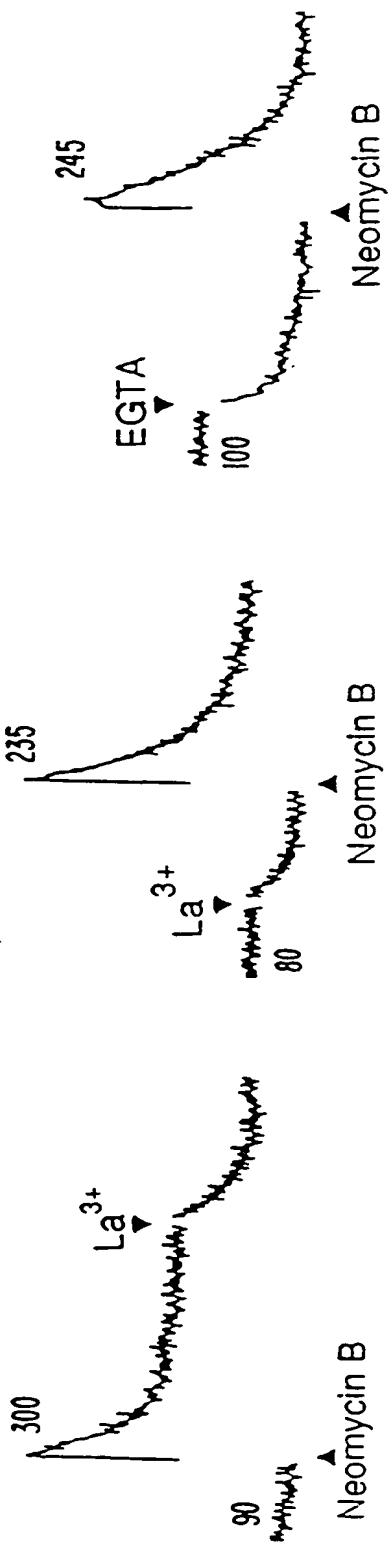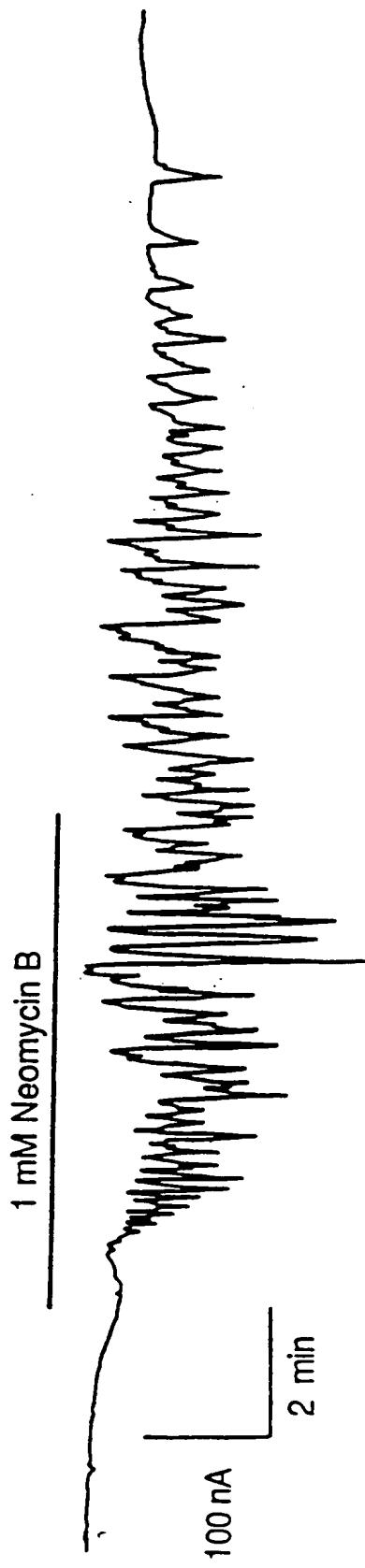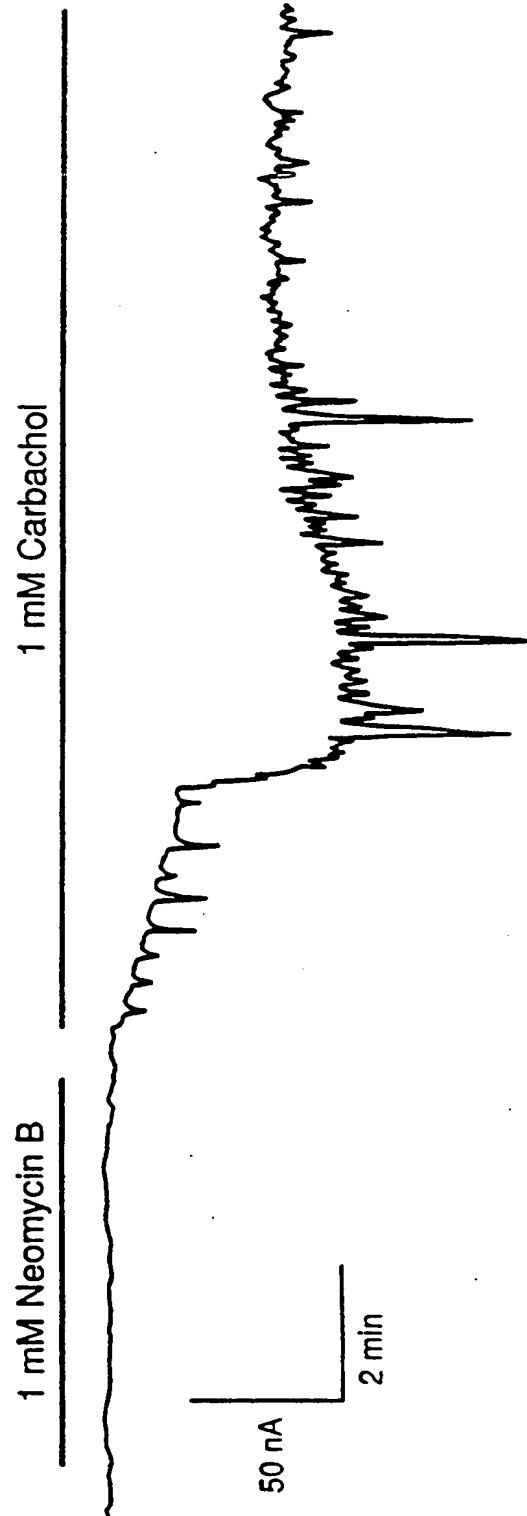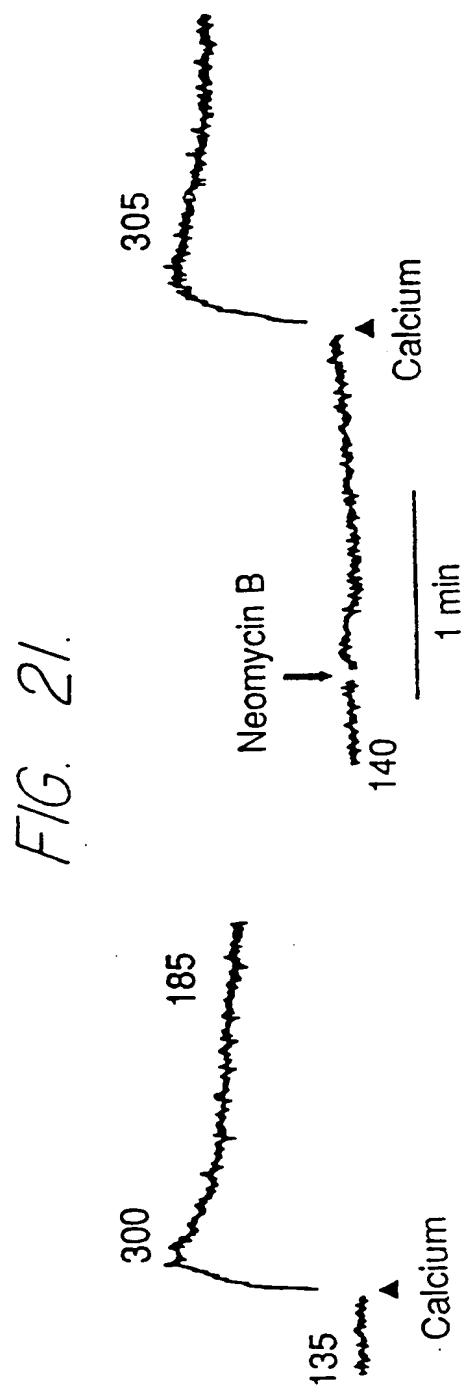
FIG. 22.

FIG. 23.

FIG. 24.

FIG. 25a.

10 mM Mg^{2+}

FIG. 25b.

600 uM Gd^{3+}

50 nA

1 min

FIG. 25c.

10 mM Mg^{2+}

100 nM Substance K

FIG. 26.

10 mM Calcium

2 min

100 nA

FIG. 27.

FIG. 28.

FIG. 29.

FIG. 30a.

(a.)
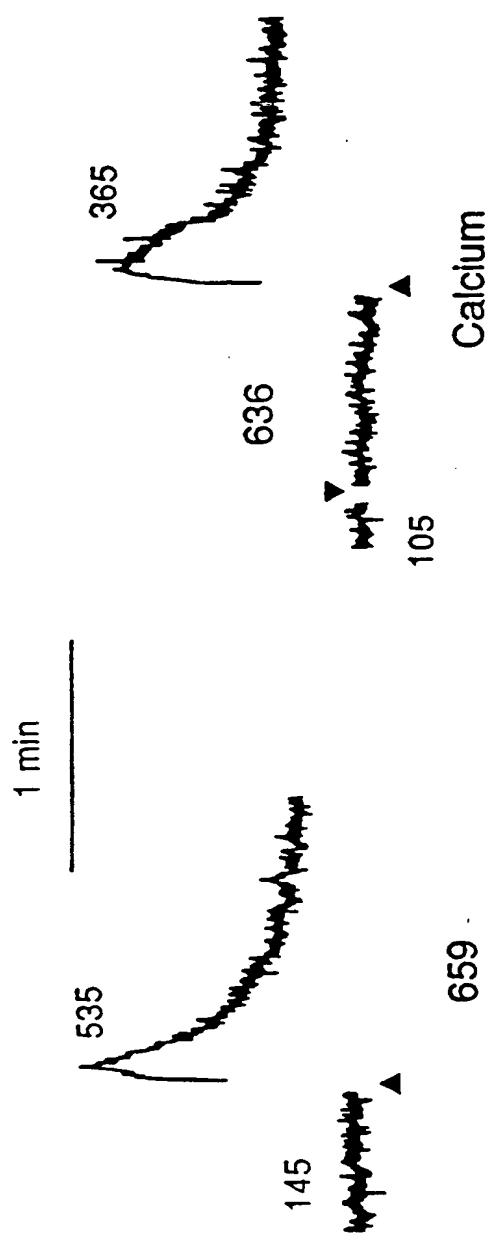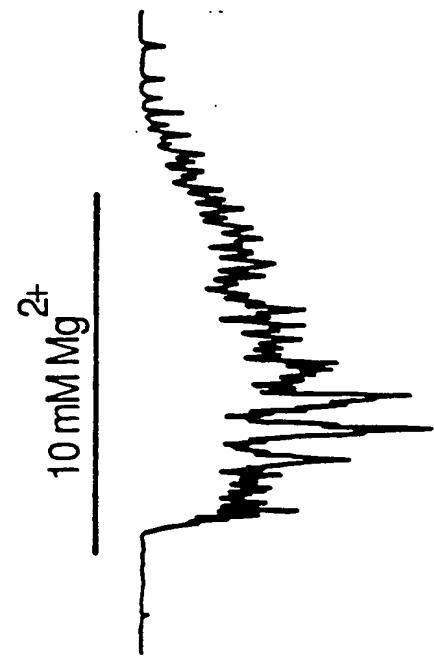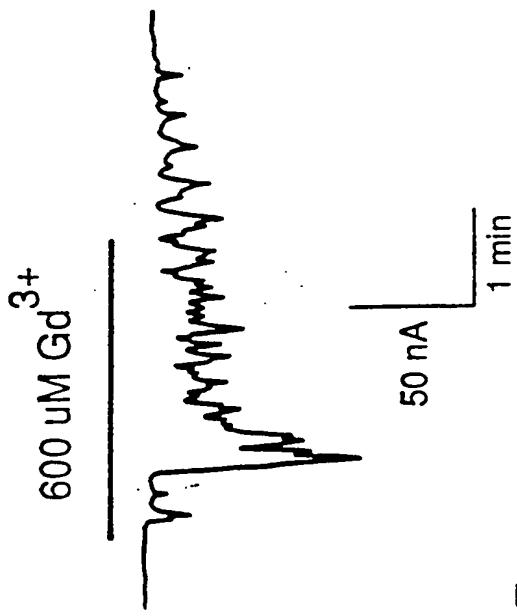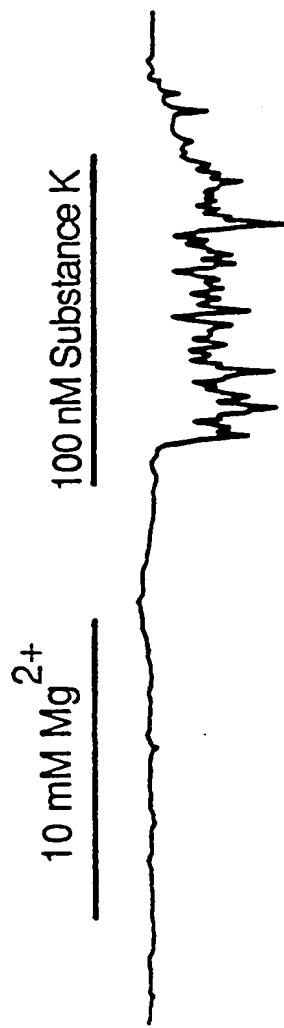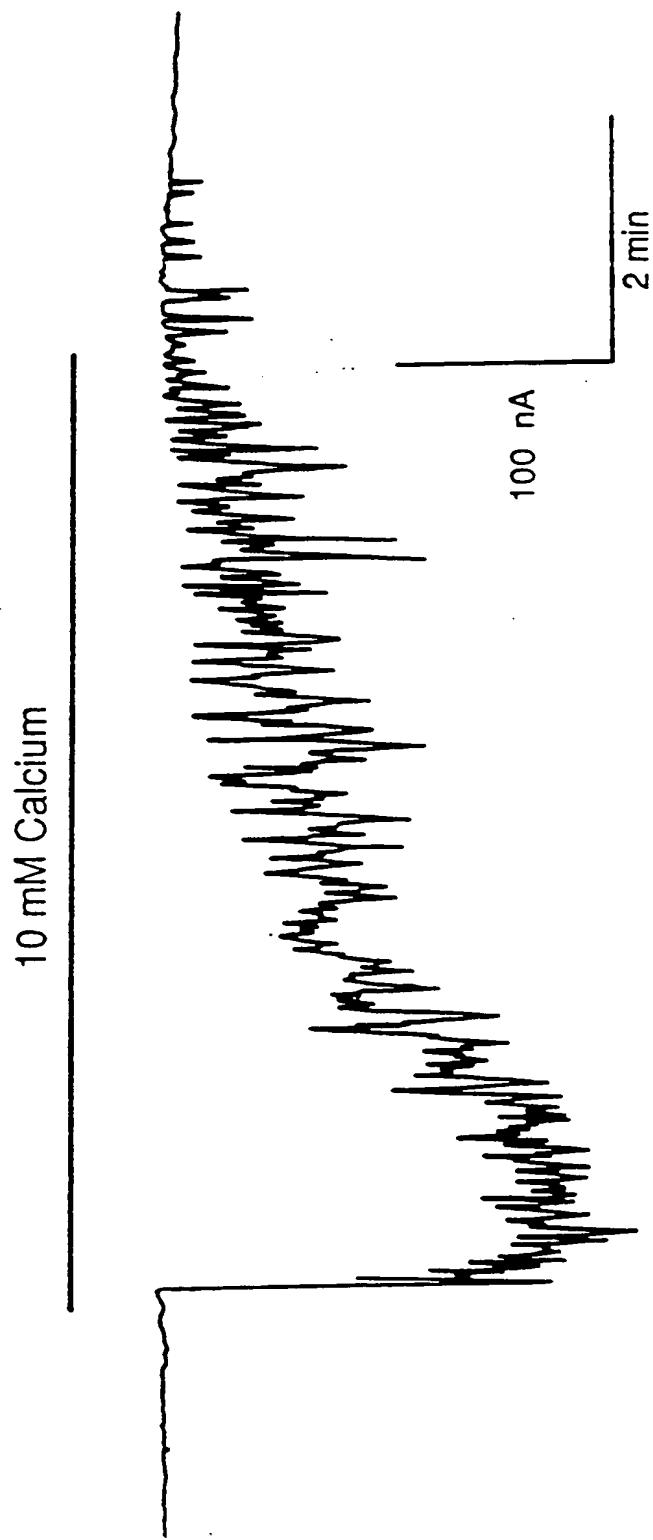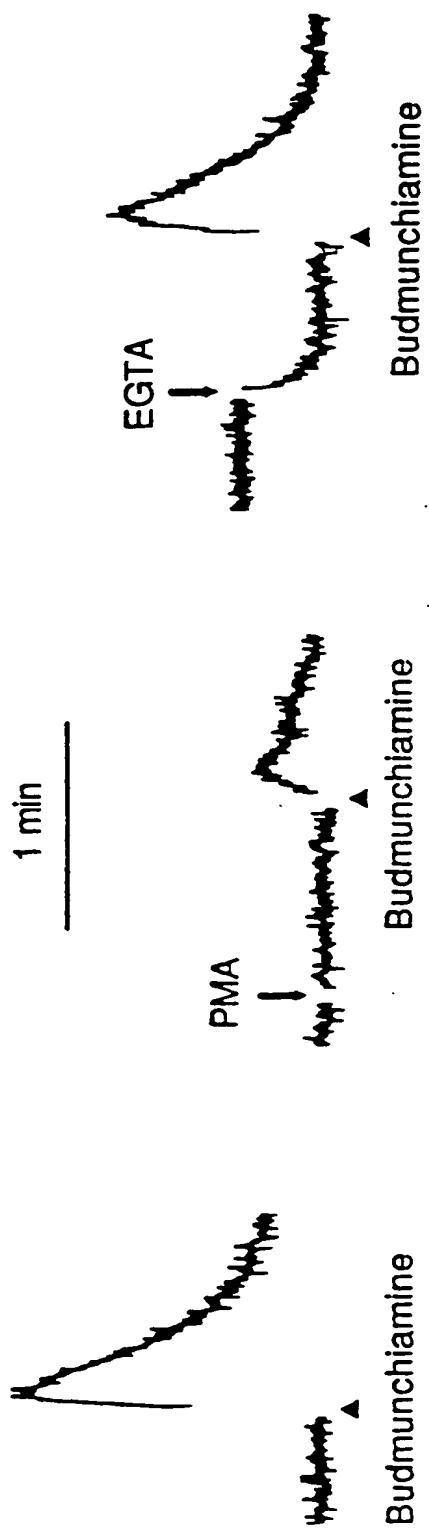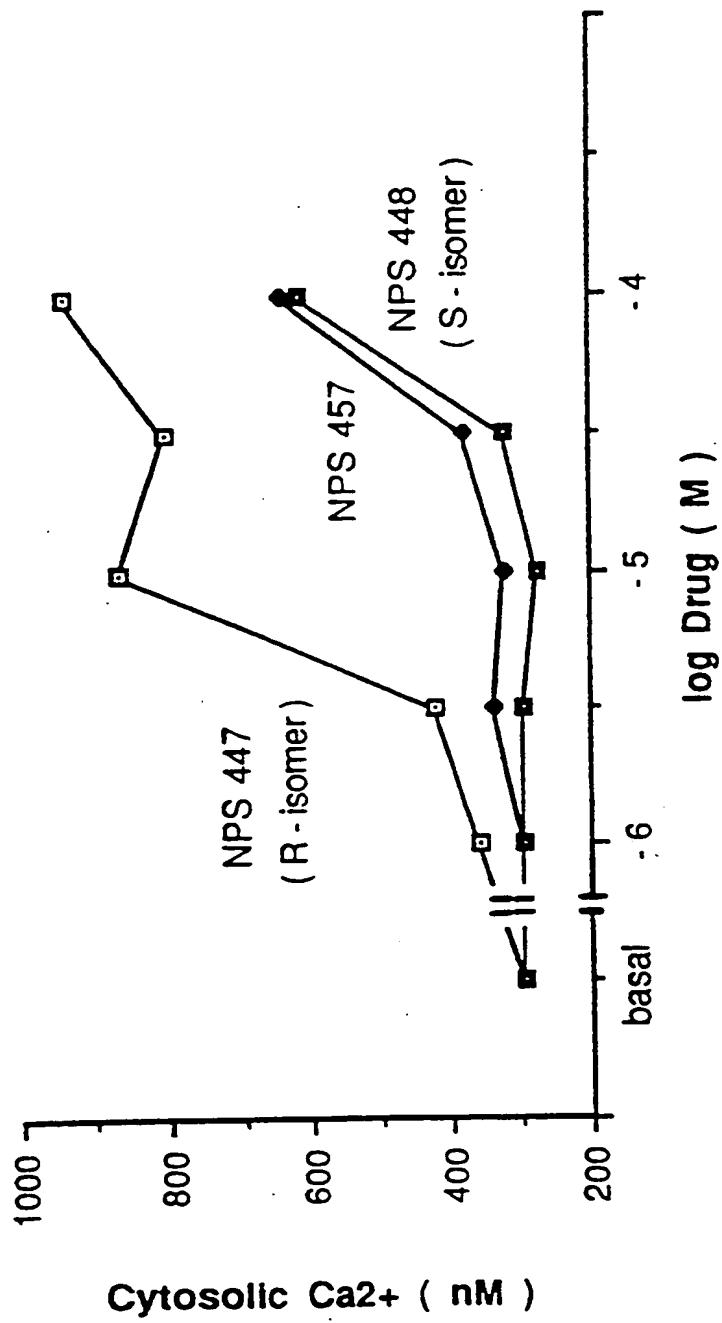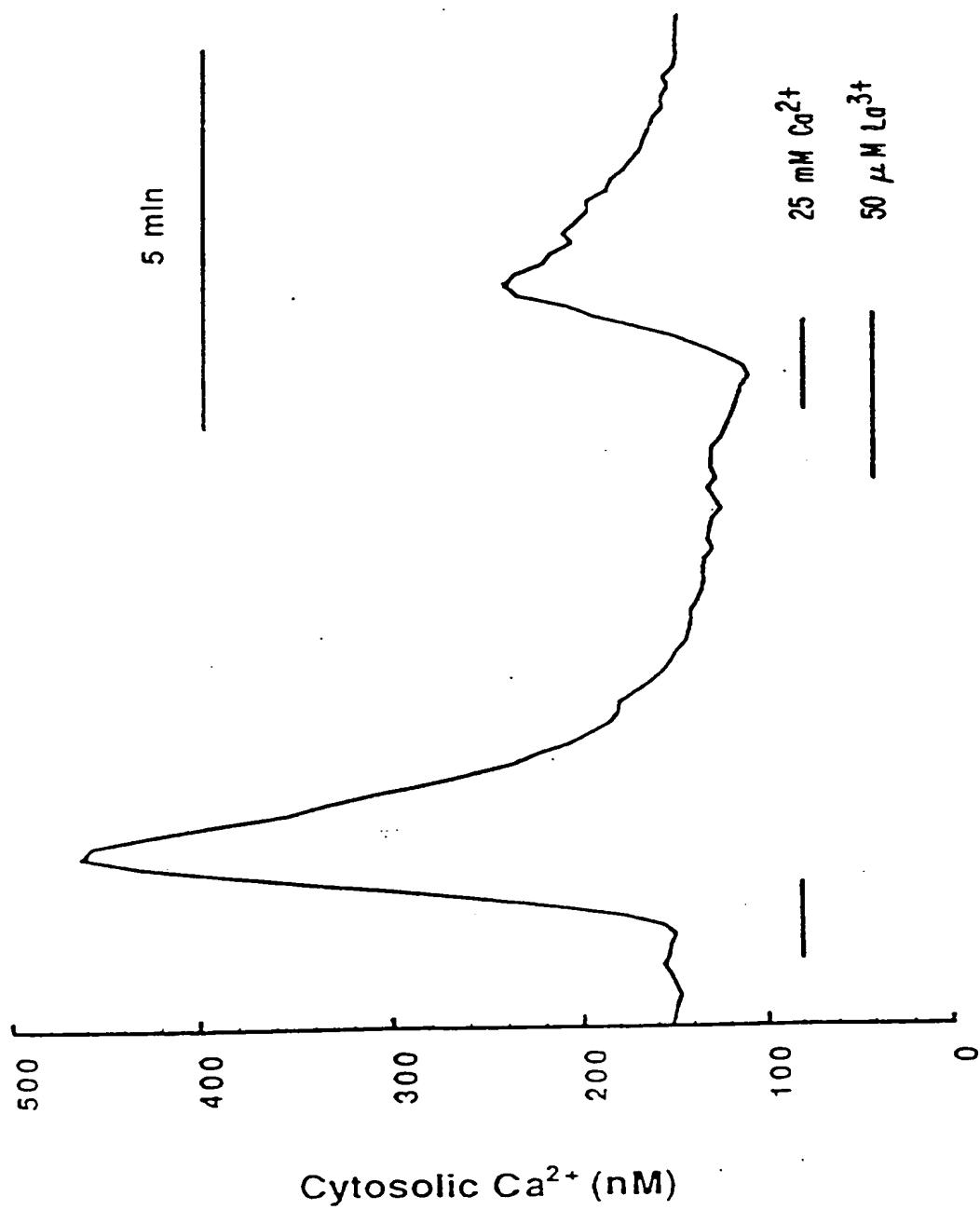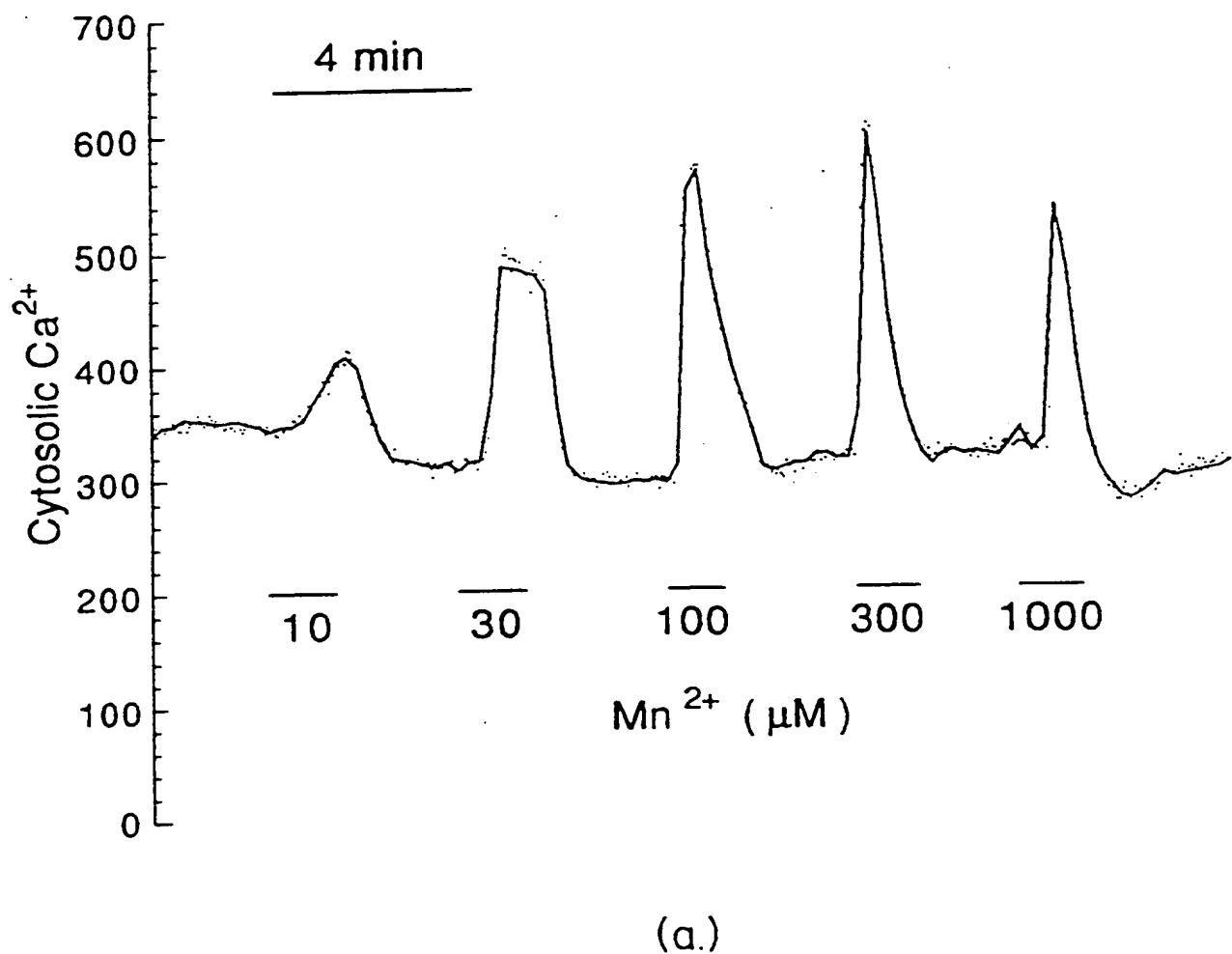
FIG. 30b.

(b.)

FIG. 3/a.

FIG. 3lb.

FIG. 32.

FIG. 33.

100 µM NPS 456

50 nA

2 min

FIG. 34.
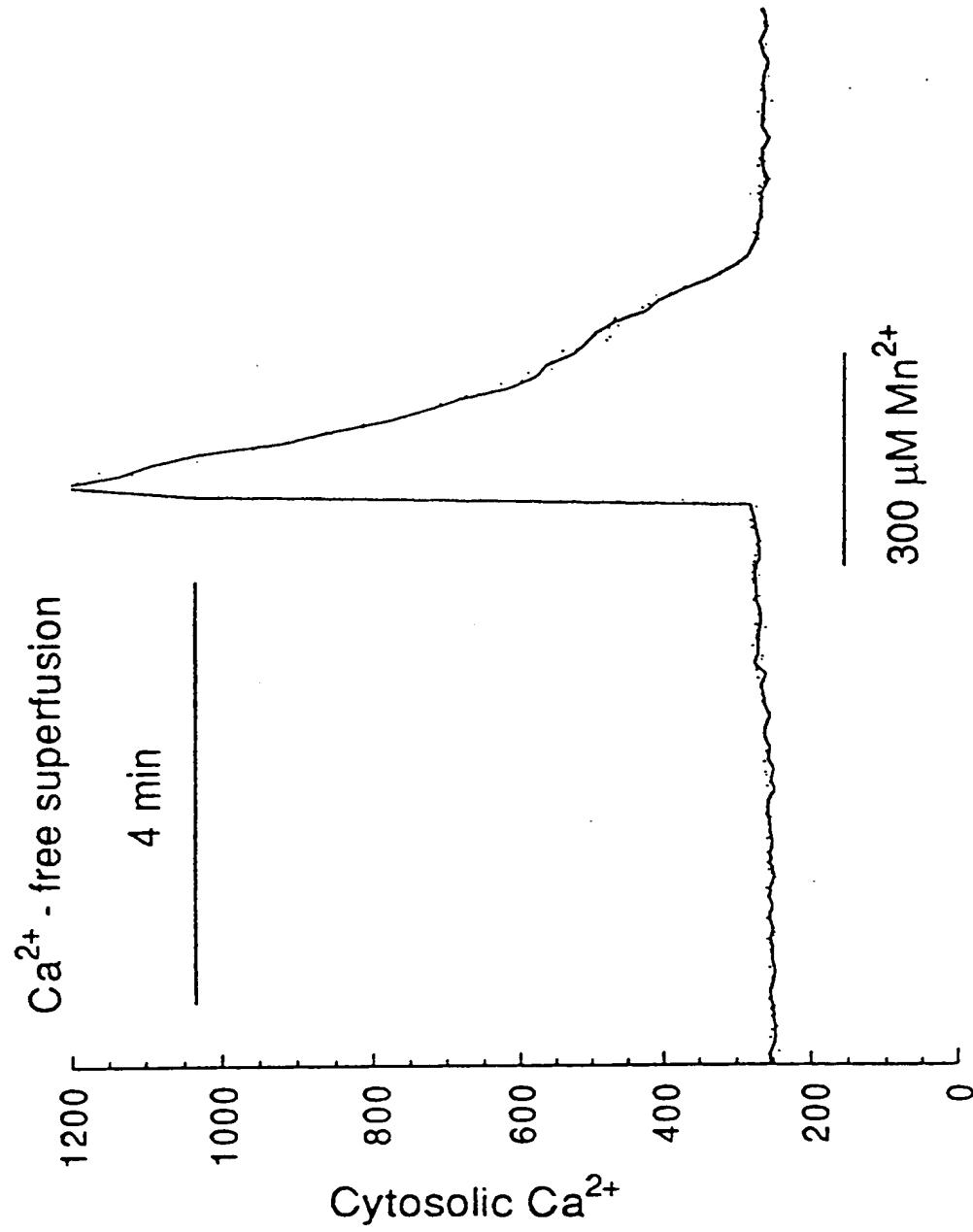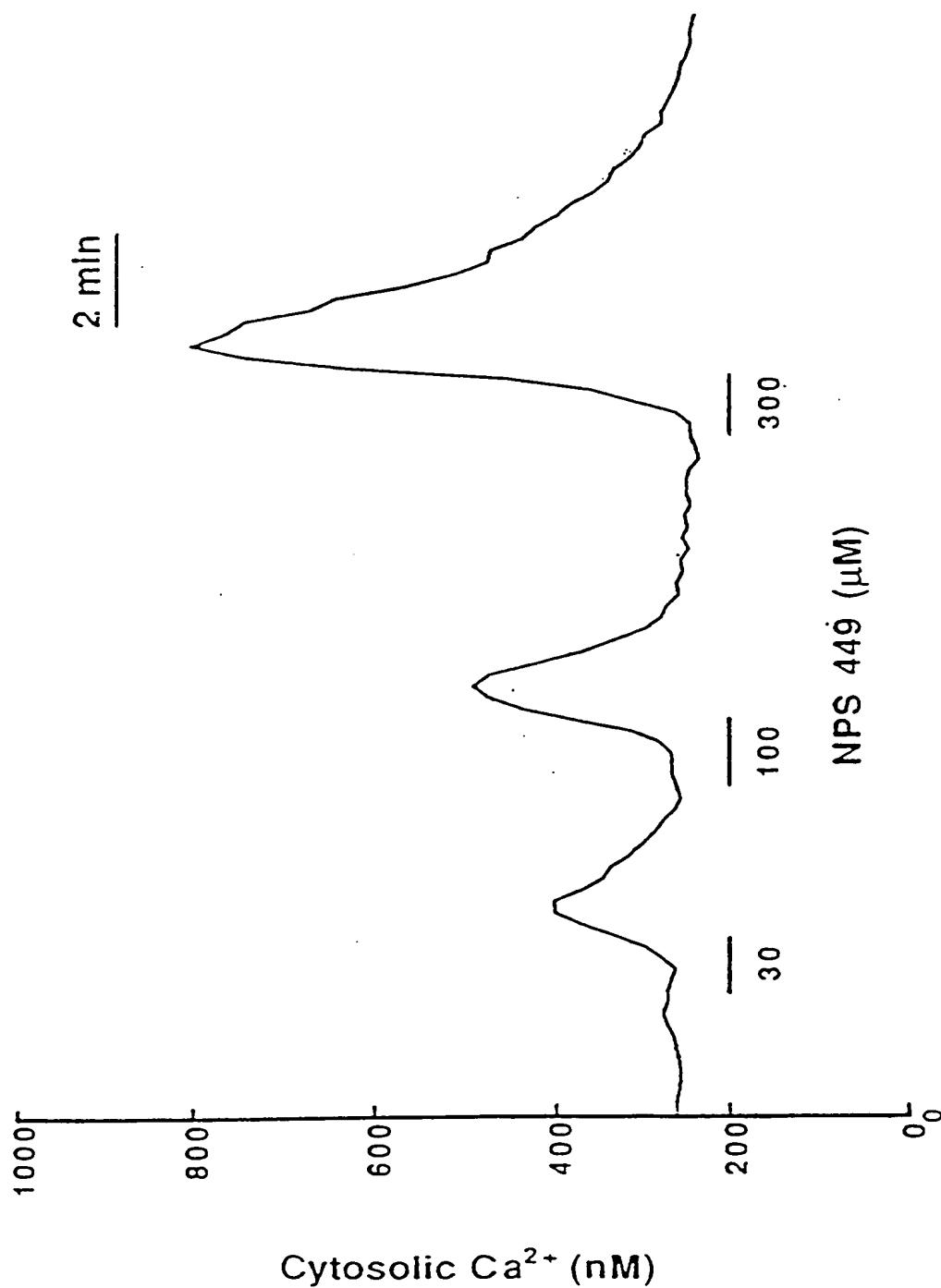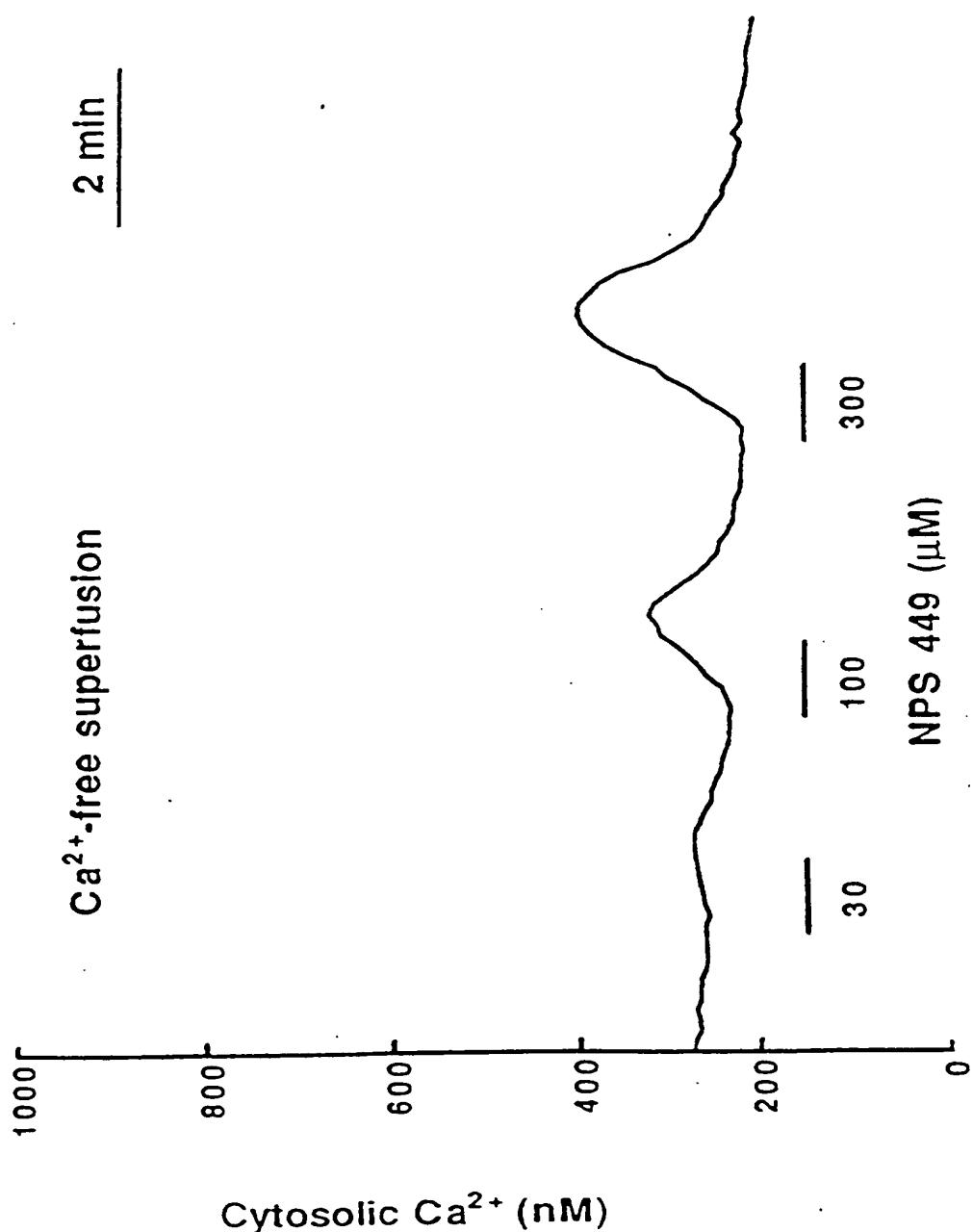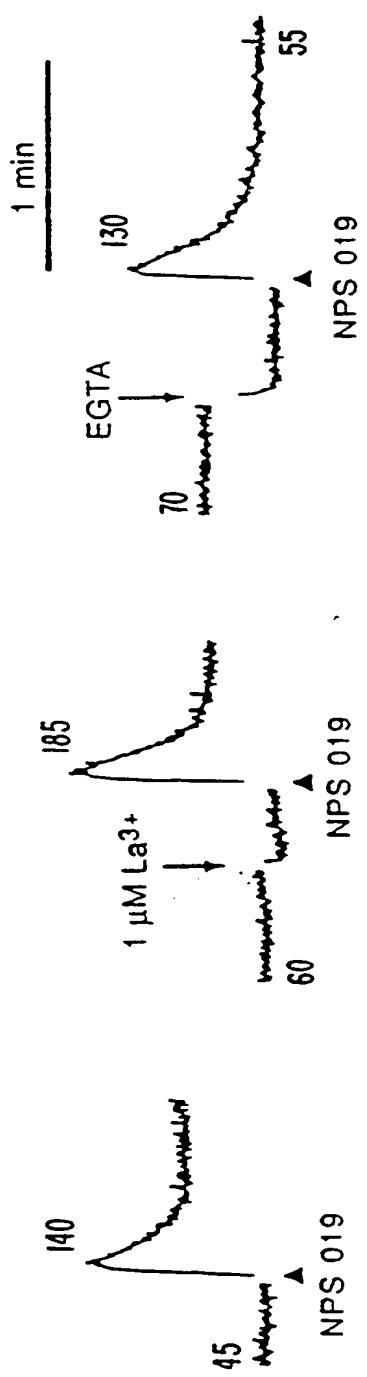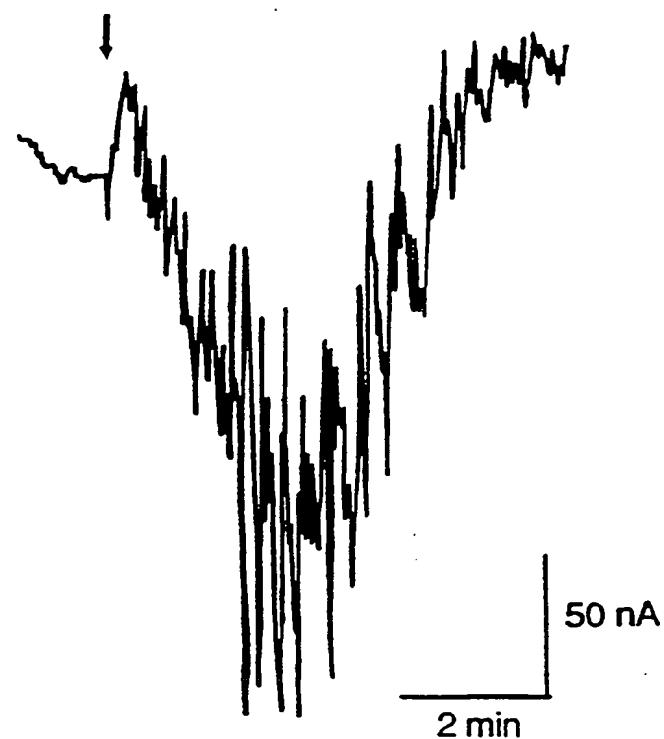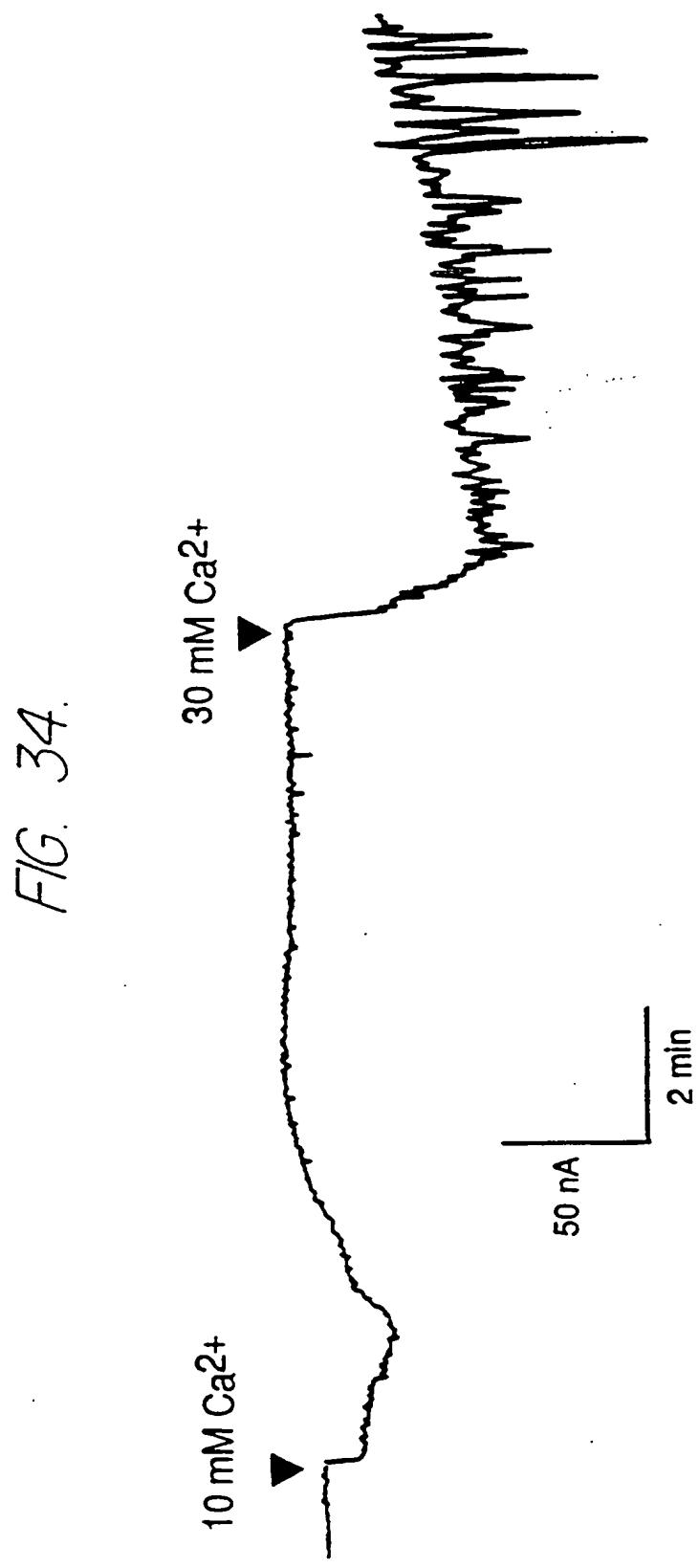
SUMMARY OF bPG mRNA FRACTION ACTIVITY
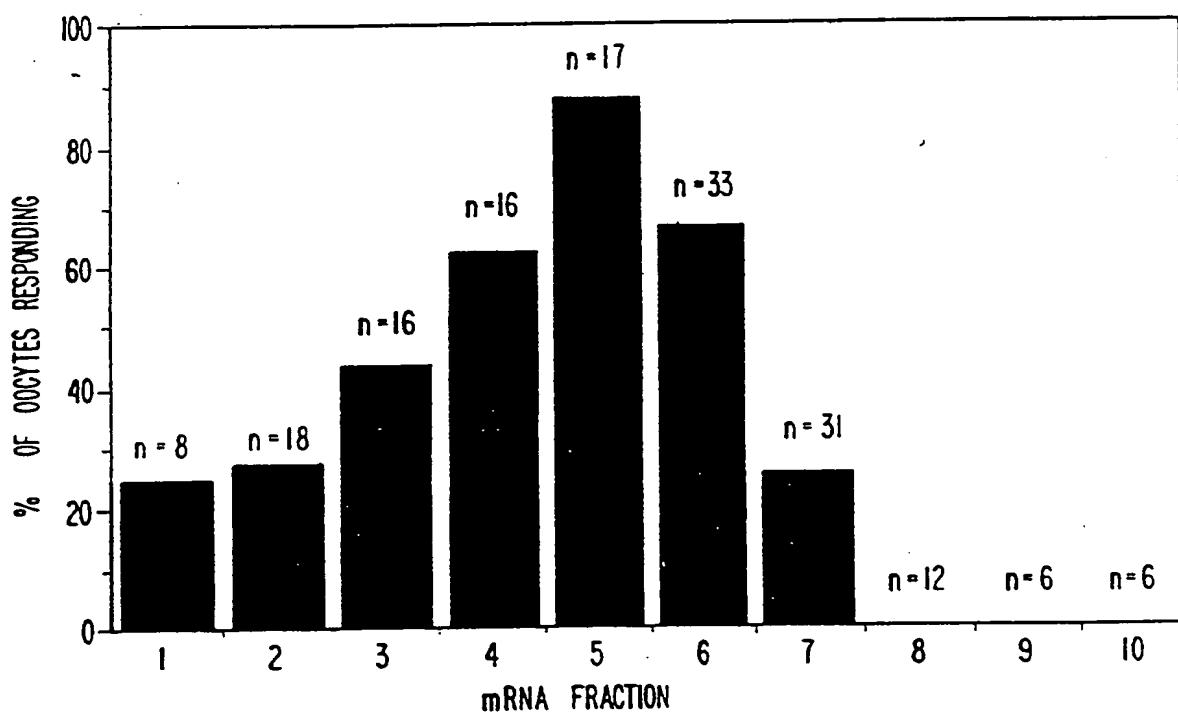
FIG. 35.
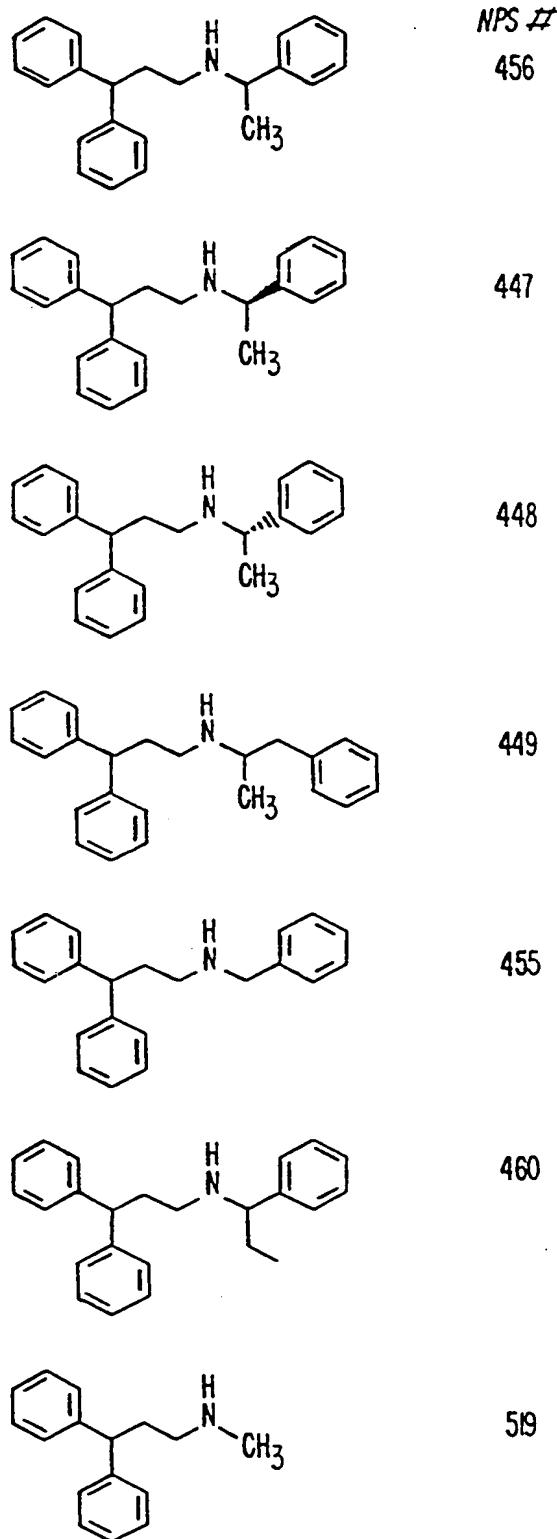
FIG. 36a.
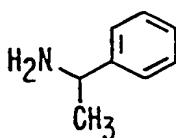
NPS #

518
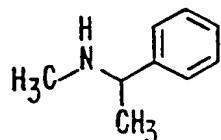
517
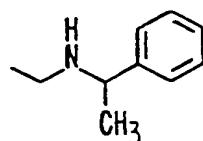
516
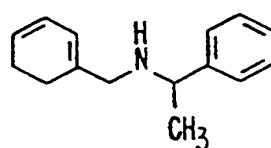
457
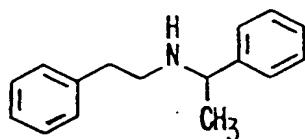
458

459

523

524
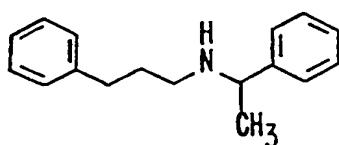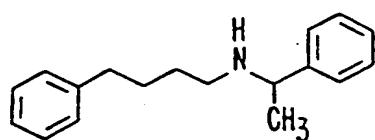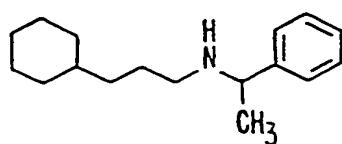
FIG. 36b.

SUBSTITUTE SHEET

NPS #
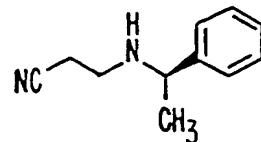
527
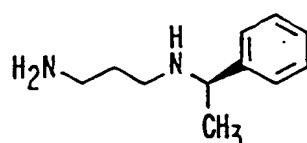
534
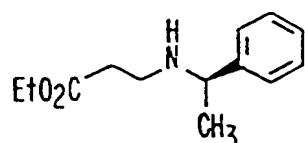
528
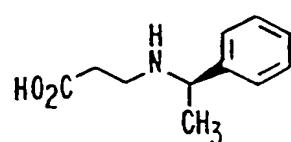
530
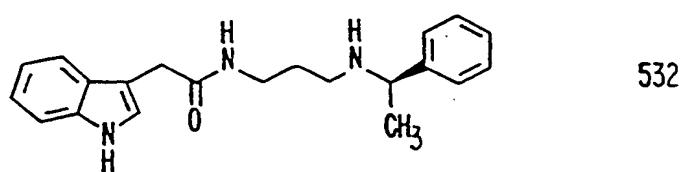
532
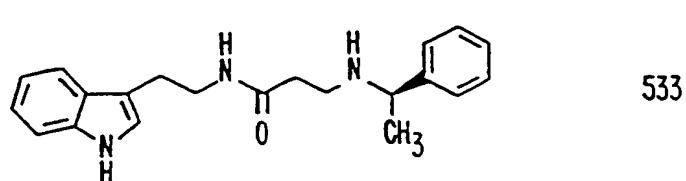
533
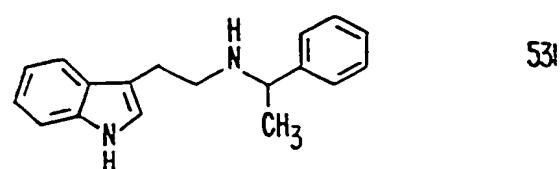
531

FIG. 36c.

NPS #
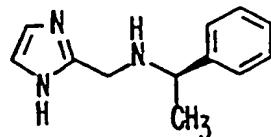
526
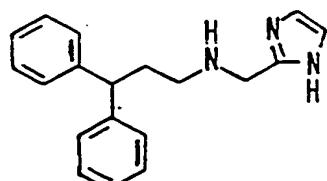
535
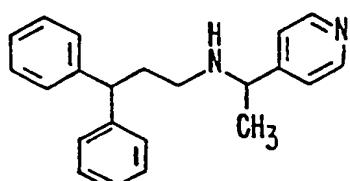
536
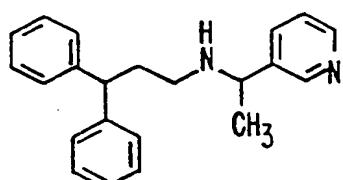
537
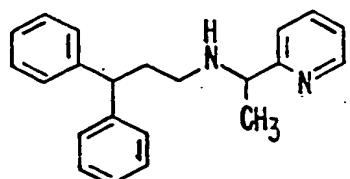
538
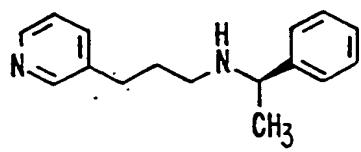
543
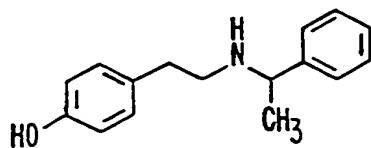
529

FIG. 36d.

SUBSTITUTE SHEET

NPS #
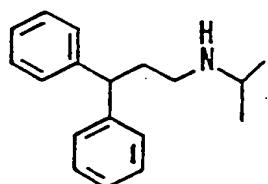
539
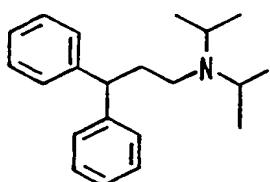
540
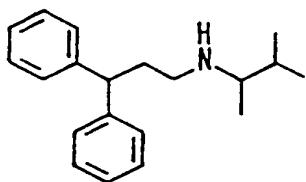
542
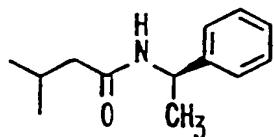
546
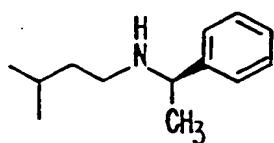
547

FIG. 36e.

NPS #
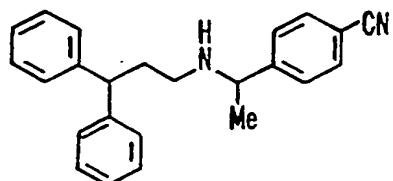
541

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FIG. 36f.

SUBSTITUTE SHEET
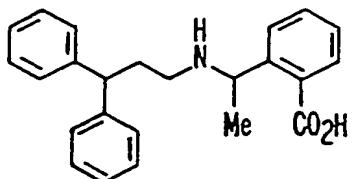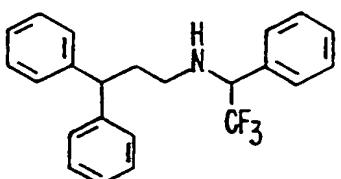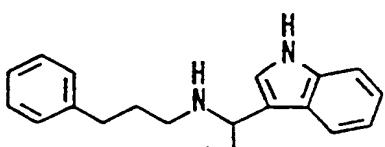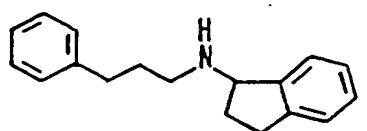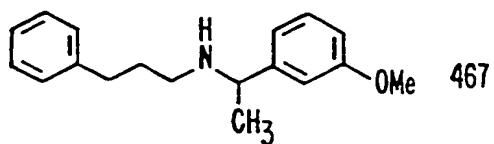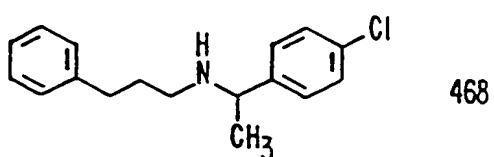
NPS #
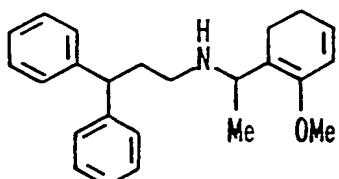
550
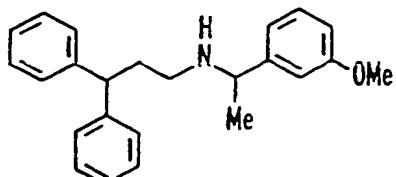
551
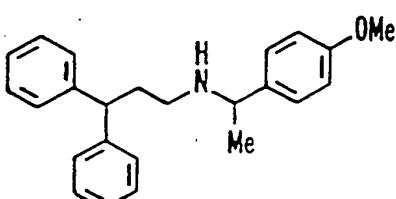
552

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FIG. 36g.

NPS #

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FIG. 36h.

SUBSTITUTE SHEET
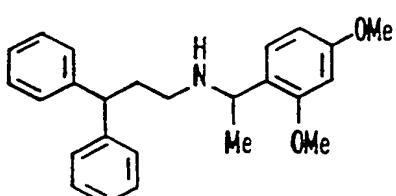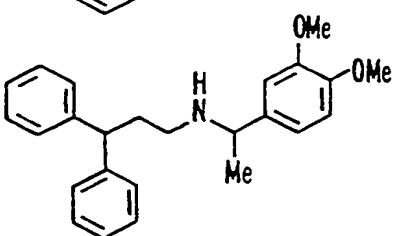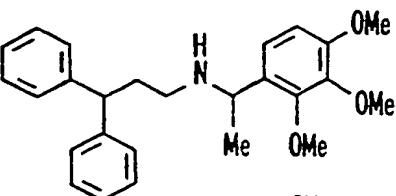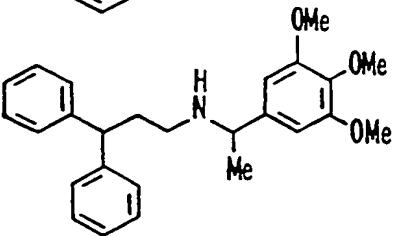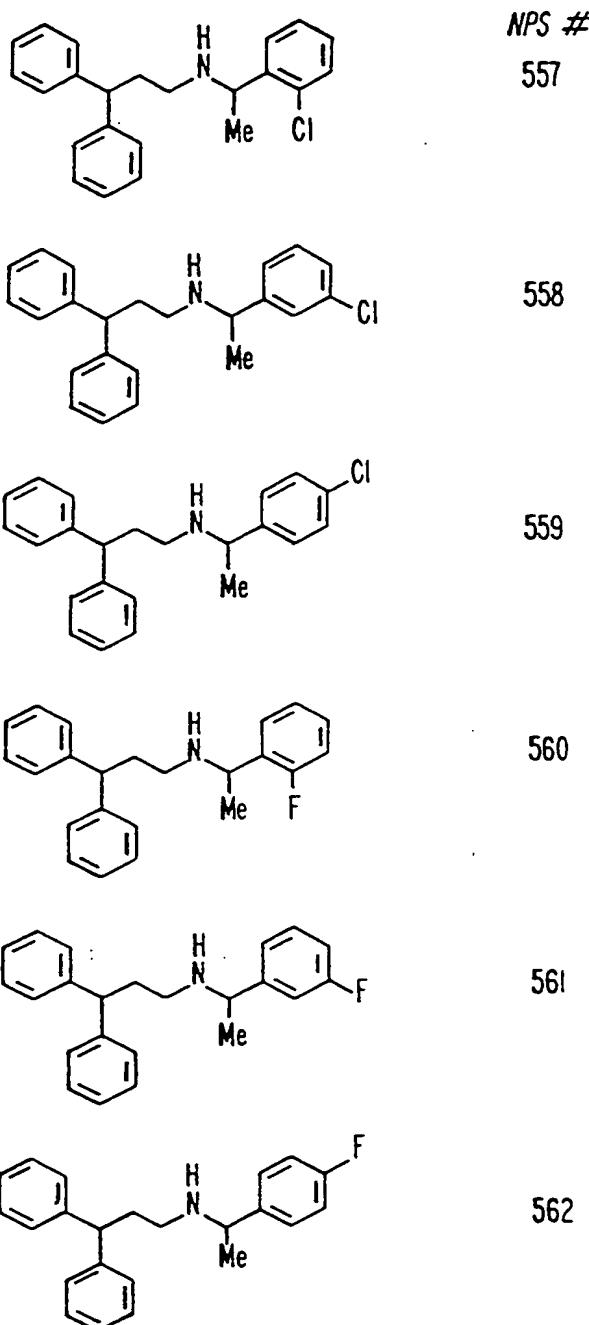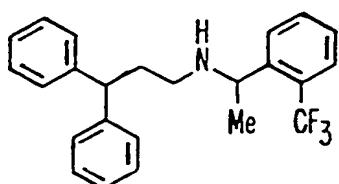
NPS #

563
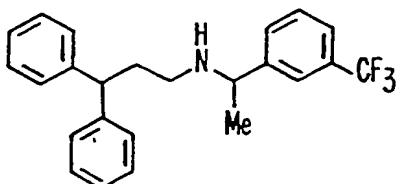
564
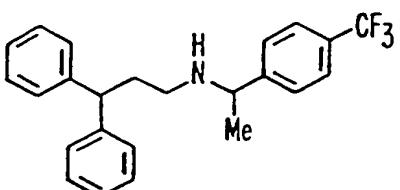
565
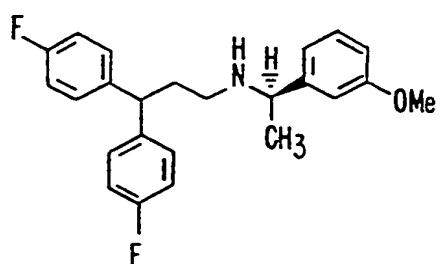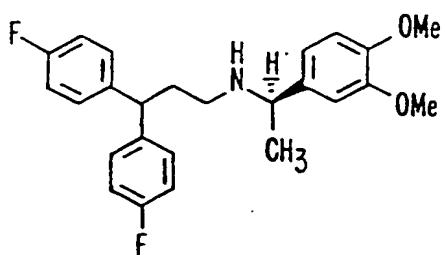
606

FIG. 36i.

NPS #
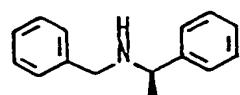
457
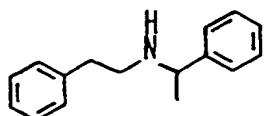
458
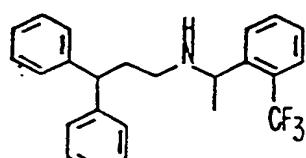
563
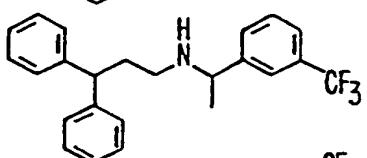
564
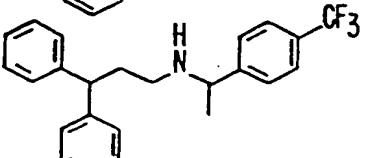
565
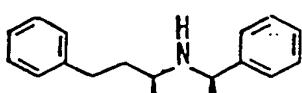
566
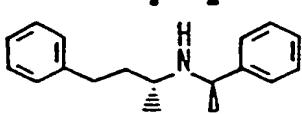
567
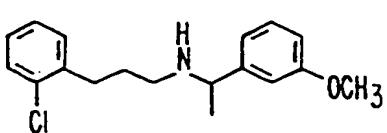
568
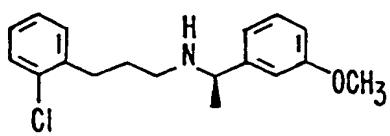
FIG. 36j.
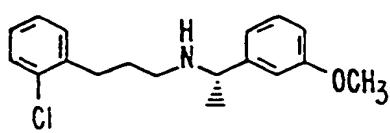
NPS #

570
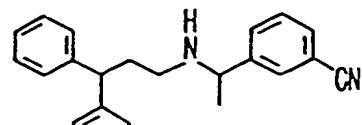
571
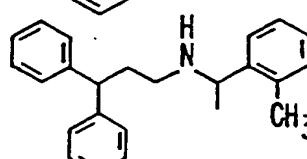
572
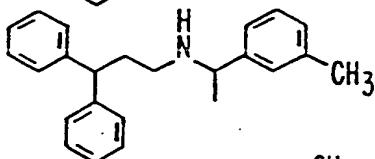
573
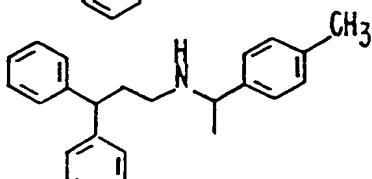
574
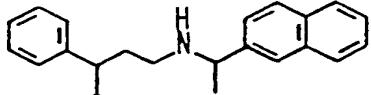
575
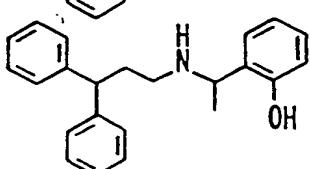
576
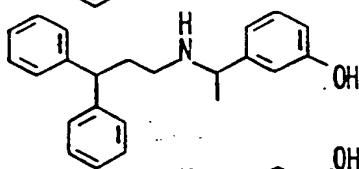
577
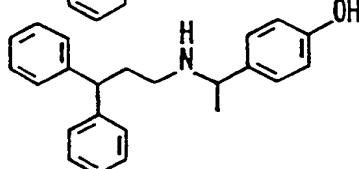
578
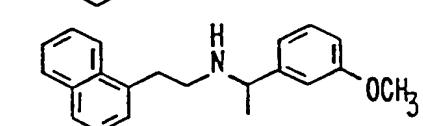
579
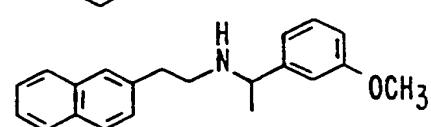
580
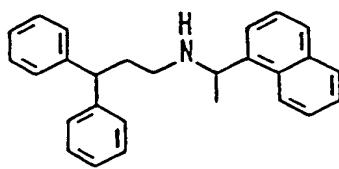
FIG. 36k.

NPS #
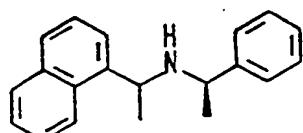
592
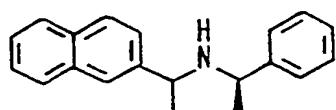
593
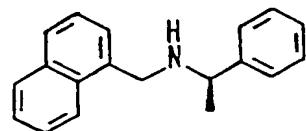
594

FIG. 36L.
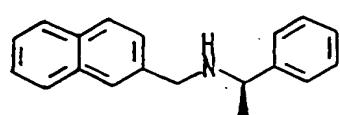
NPS #

595
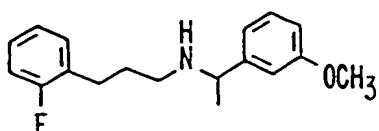
601
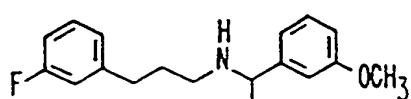
602
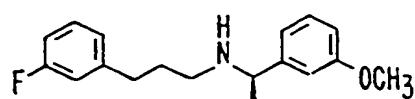
603

604

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607
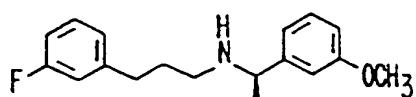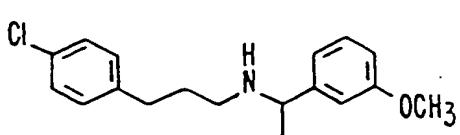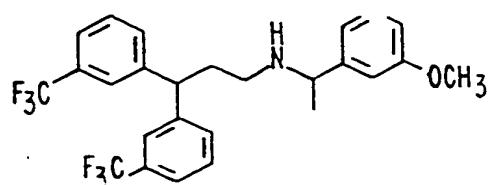
FIG. 36m.

SUBSTITUTE SHEET

NPS #
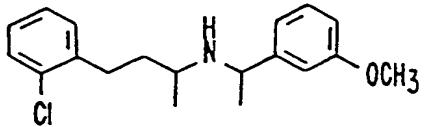
609
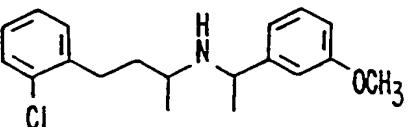
610
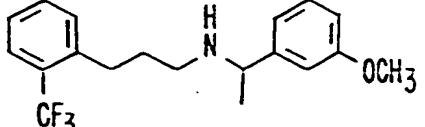
611
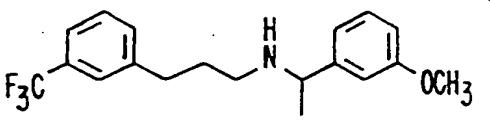
612
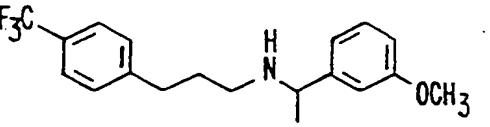
613
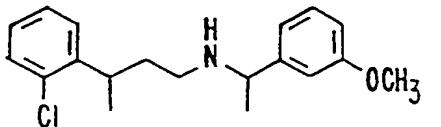
614

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620
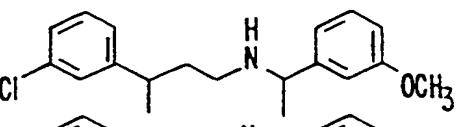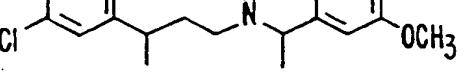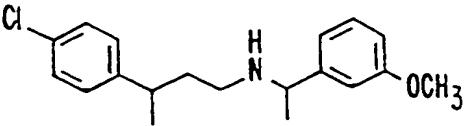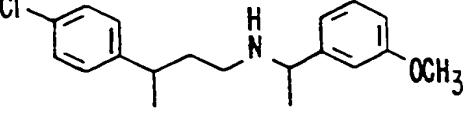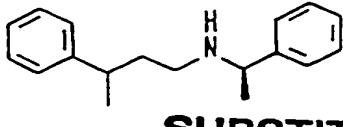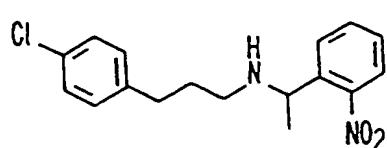
FIG. 36n.

NPS #

623
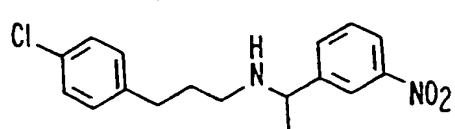
624
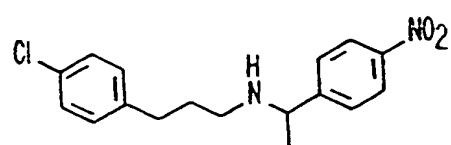
625
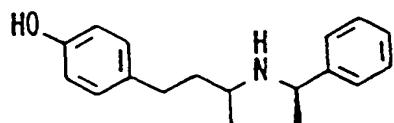
631
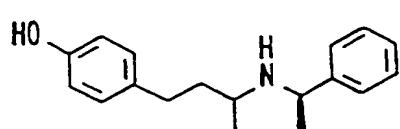
632

FIG. 360.

SUBSTITUTE SHEET

NPS #

641

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FIG. 36p.

60/74
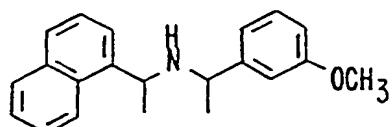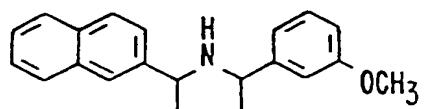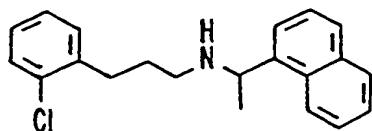
NPS #

646
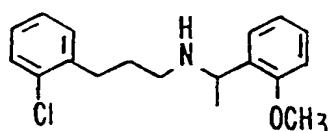
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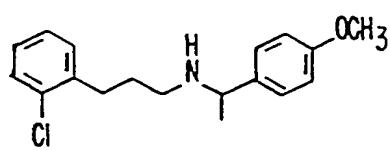
652

653

654

655

FIG. 36g.

SUBSTITUTE SHEET
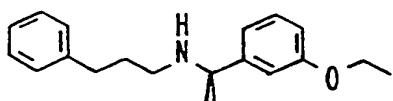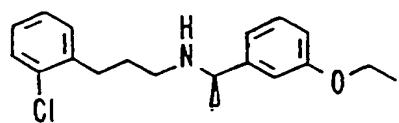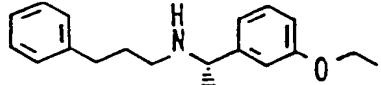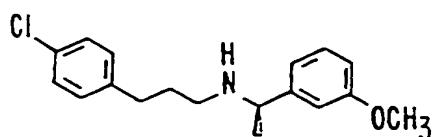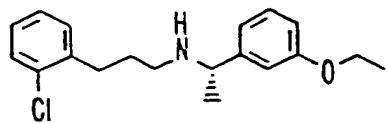
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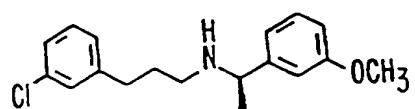
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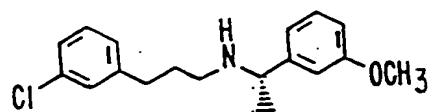
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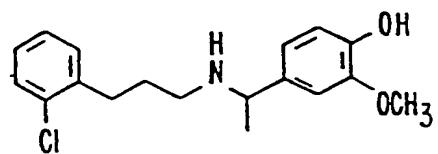
FIG. 36r.

NPS #
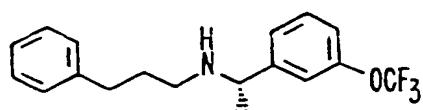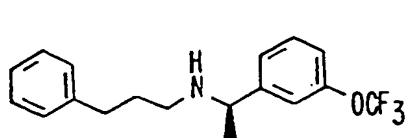
689
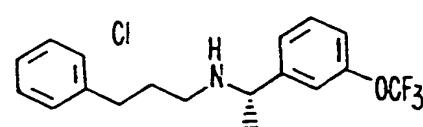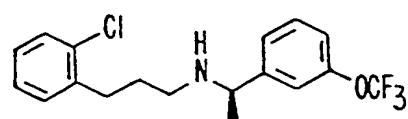
691

706

708

709

FIG. 36s.

NPS #

710

711

FIG. 36t.

FIG. 37b.

205

185

Ca2+

200 μM
NPS021

120

FIG. 37a.

620

480

Ca2+

105
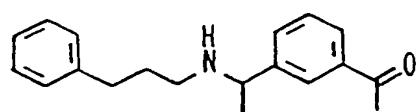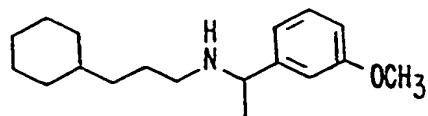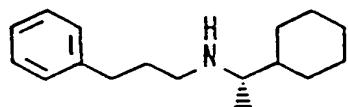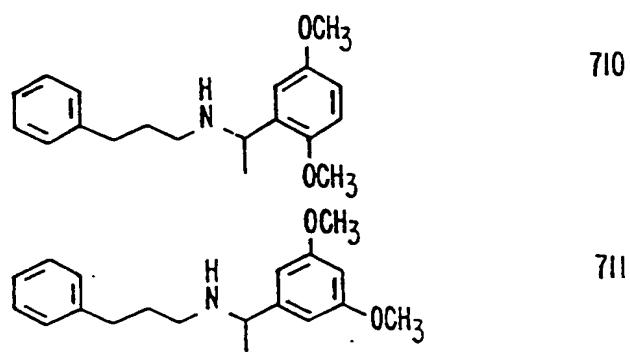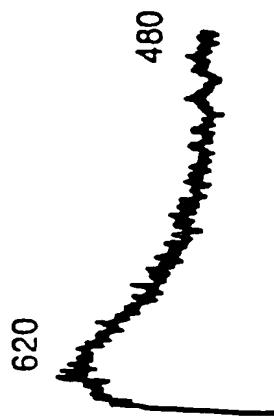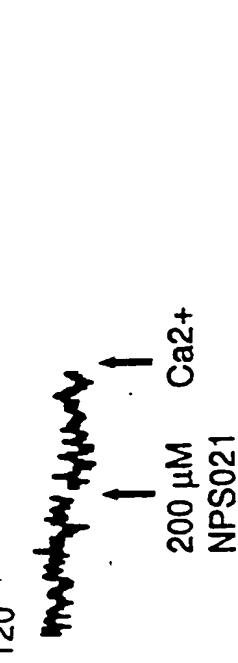
FIG. 38

Ionized Calcium Response to NPS 467

PTH RESPONSE TO 467

FIG. 39.

FIG. 40.

Ionized Calcium Response to 25 mg/kg 467

FIG. 41.

DE vs LE ISOMERS OF NPS467 ——————— EFFECT ON IONIZED CALCIUM
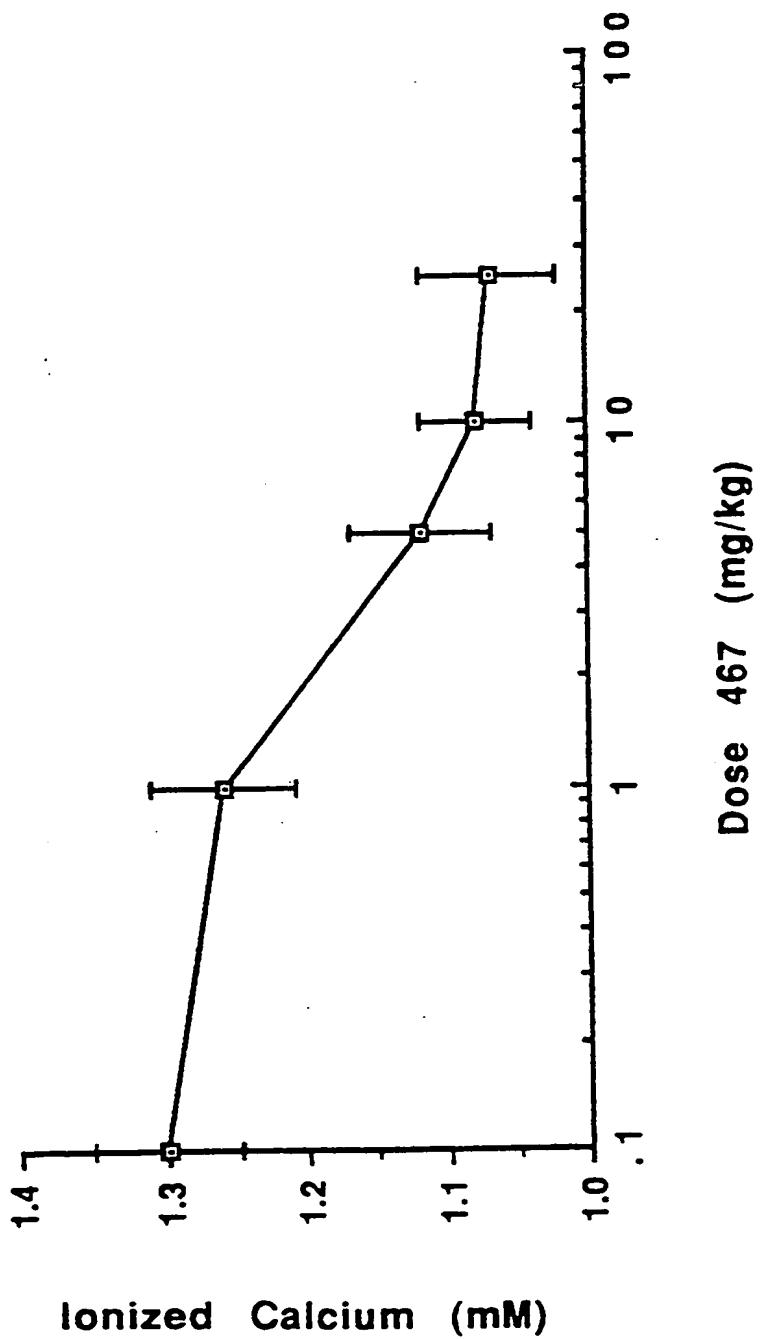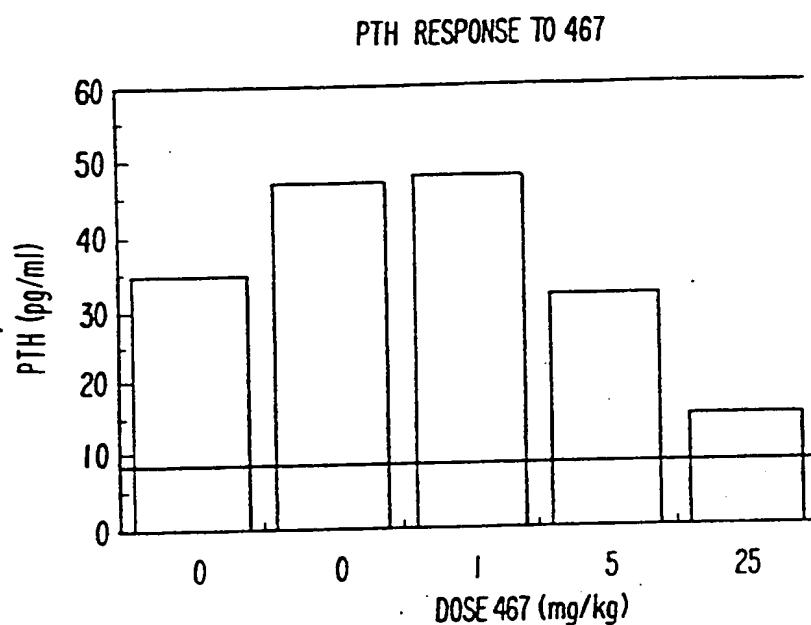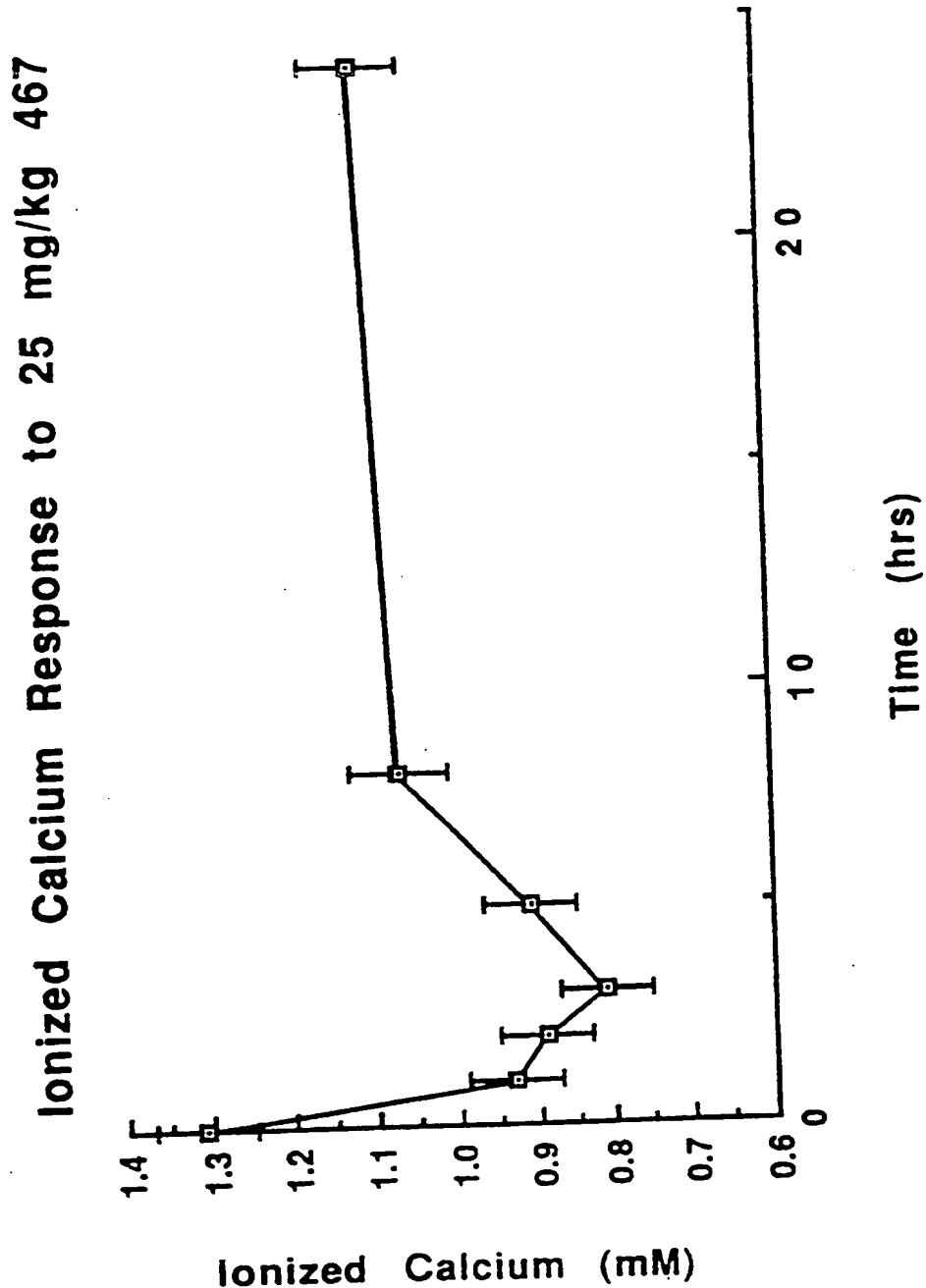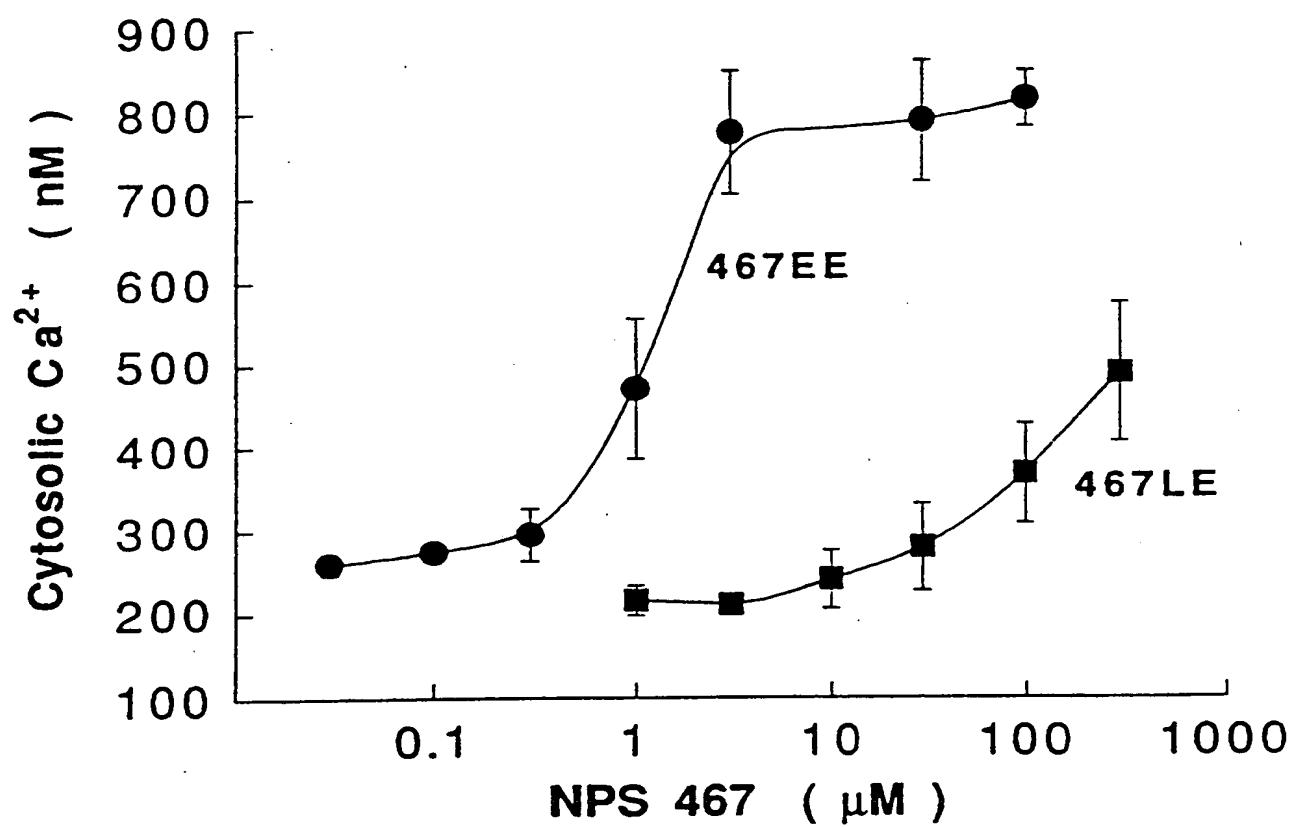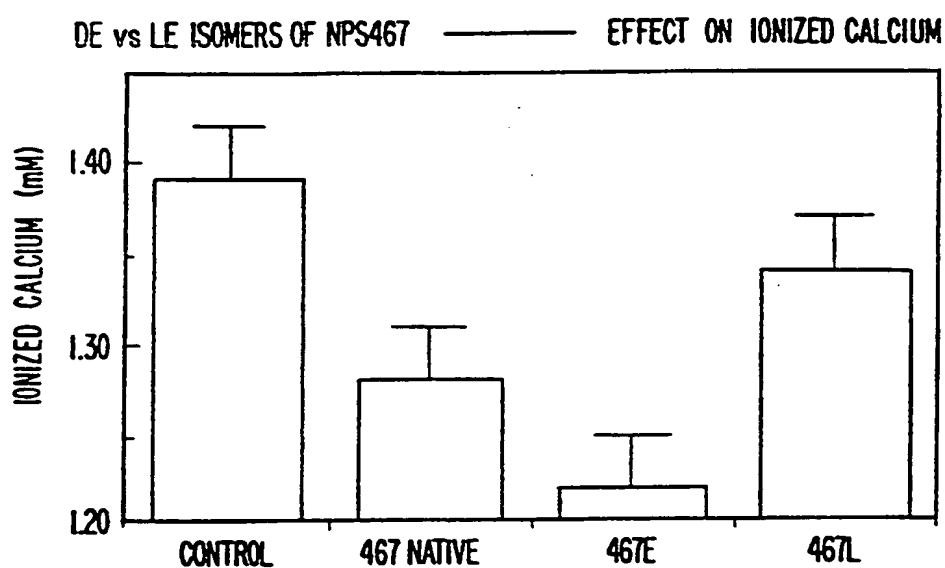
FIG. 42.
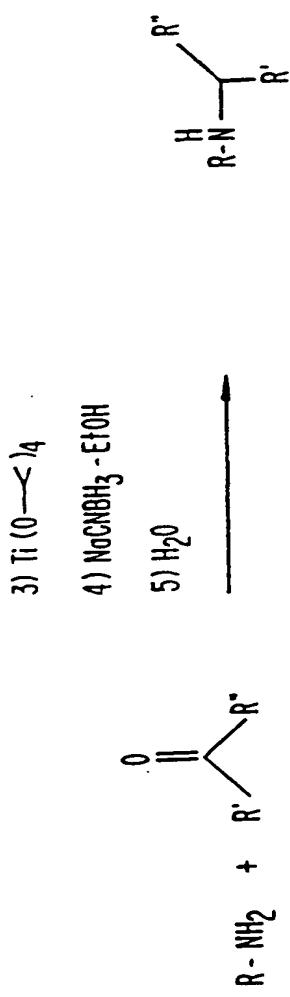
FIG. 43a.

FIG. 43b.
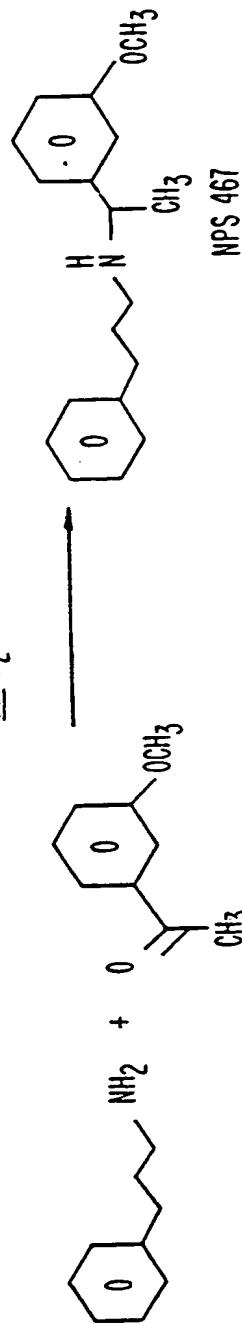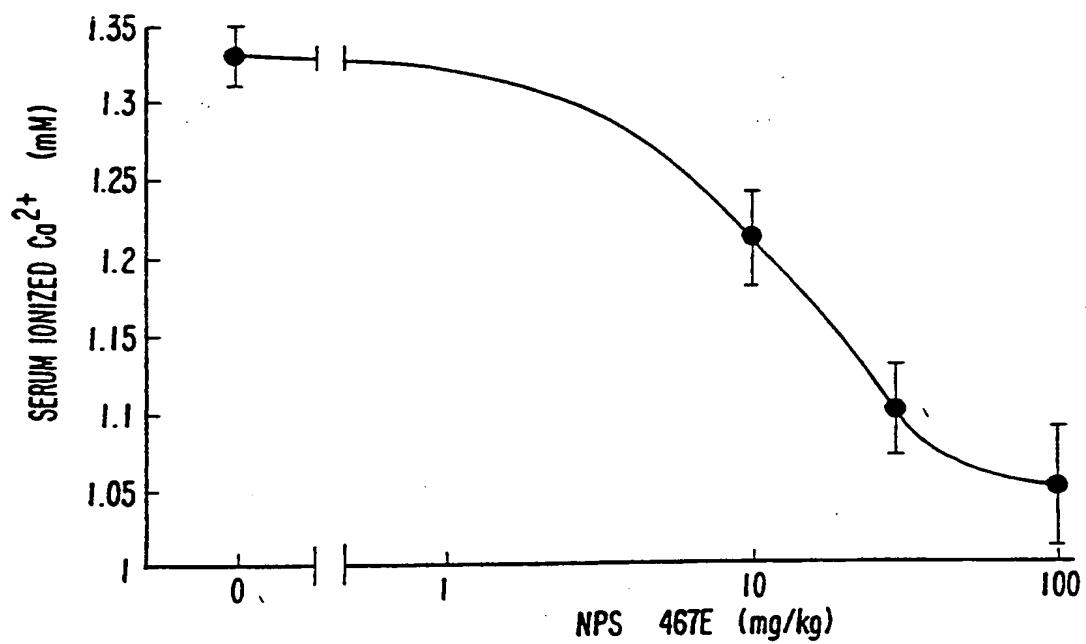
FIG. 44.

BoPCaR1 cDNA Restriction Map
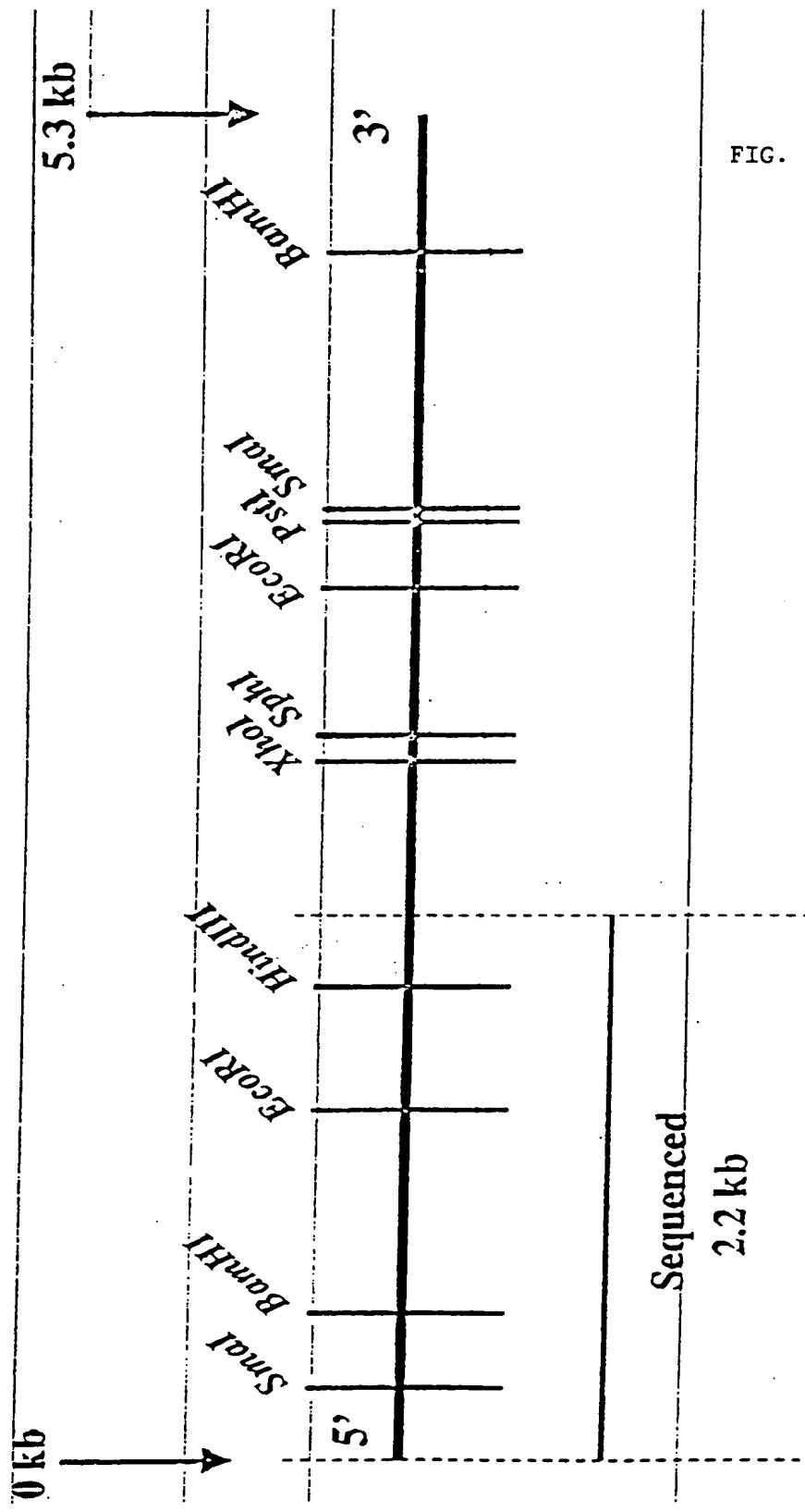
FIG. 45
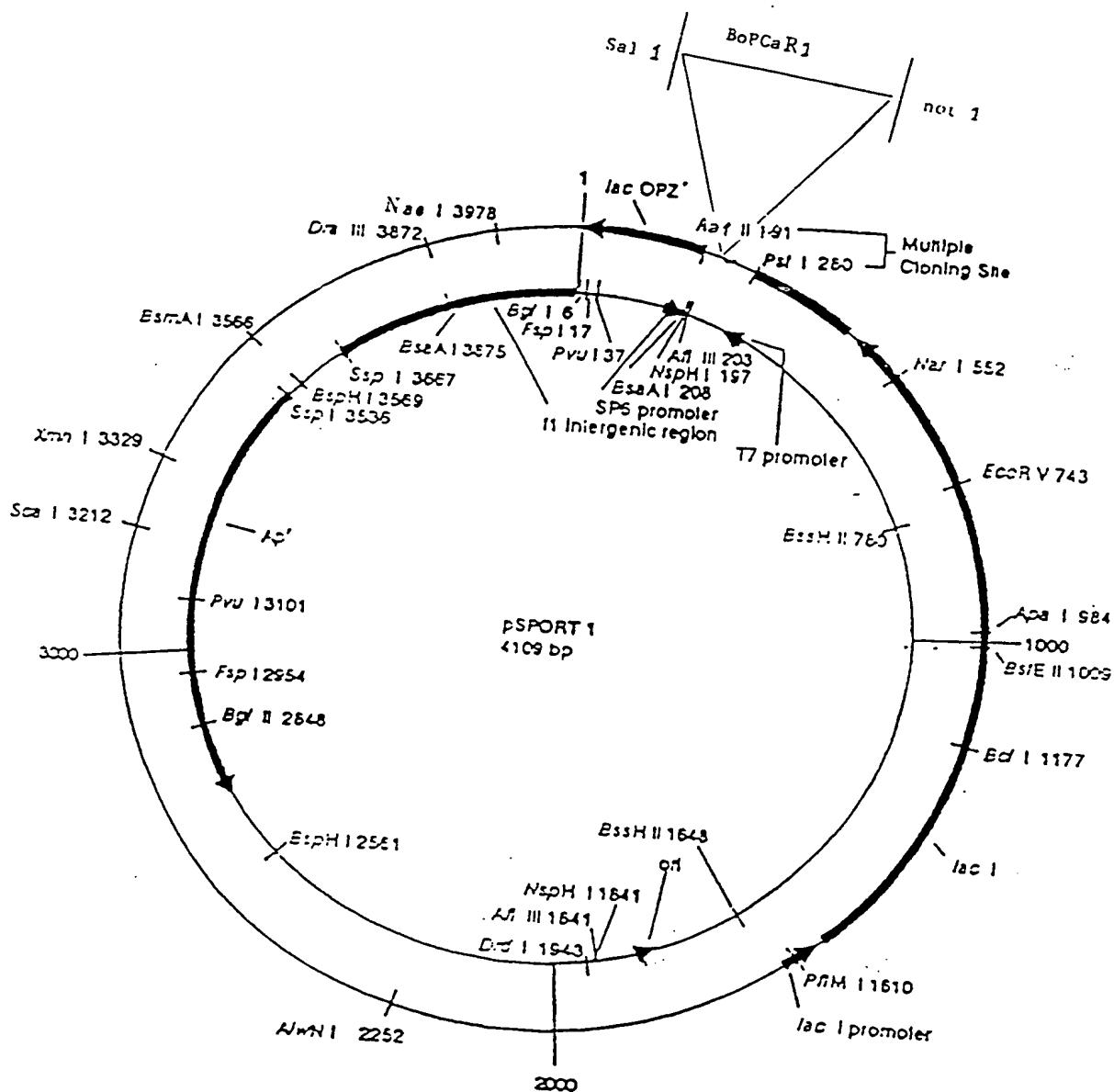
FIG. 46

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   1 CCACGCGTCCGCGGA AAAAAAAAAAAAGTT CCCCACTCTAGTACA GAGAAGGTTGGCAGA

  60 GTCGTAAGCCCCCAA CCTCTTAAACTTCTC TGCATCTCCAAGGAG AAGGAGGGAAGAGGG
 120 GTTCTTTCCGACCTG AGGAGCTGGATCTGG GGTCCGAGAACCCCA AGGTAGCACCGGAAA
 180 GAACAGCACAGGAGG CGAGAGCGTGCGGTG GCCGGTKGGCGGGAG AACCAGACCCGACKY
 240 CGCGGTCCTCGGYGC TGCCGGGKYCCSGGG ACTCAGCTCAGCACG ACTGGGAAGCCGAAA
 300 GTACTACACACGGTC TCTGCATGATGTGAC TTCTGAAGACTCAAG AGCCACCCACTTCAC
 360 TAGTCTGCAATGGAG AAGGCAGAAATGGAA AGTCAAACCCCACGG TTCCATTCTATTAAT
 420 TCTGTAGACATGTGC CCCCACTGCAGGGAG TGAGTCGCACCAAGG GGGAAAGTCCTCAGG
 480 GGCCCCCAGACCACC AGCGCTTGAGTCCCT CTTCCTGGAGAGAAA GCAGAACTATGGCAC
 540 TTTATAGCTGCTGTT GGATCCTCTTGGCTT TTTTCTACCTGGTGC ACTTCCGCCTATGGG
 600 CCTGACCAGCGAGCC CAAAAGAAAGGGGAC ATTATCCTCGGGGGG CTCTTTCCTATTCAT
 660 TTTGGGGTTGMMGTG AAAGATCAGGATCTA AAGTCGAGGCCGGAG TCCGTGGAGTGTATC
 720 AGGTATAATTTCCGA GGATTTCGCTGGTTA CAAGCTATGATATTT GCCATAGAGGAAATA
 780 AACAGCAGTCCAGCC CTTCTTCCCAACATG ACCCTGGGATACAGG ATATTCGACACTTGT
 840 AACACCGTCTCTAAA GCCTTGGAGGCCACC CTGAGTTTTGTGGCC CAGAACAAAATTGAC
 900 TCTTTGAACCTTGAT GAGTTCTGCAACTGC TCAGAGCACATCCCC TCTACCATCGCAGTG
 960 GTGGGAGCTACTGGC TCGGGCATCTCCACA GCAGTGGCCAACCTG CTGGGGCTCTTCTAC
1020 ATCCCCCAGGTCAGC TATGCCTCCTCCAGC AGACTCCTCAGCAAC AAGAATCAATTCAAG
1080 TCCTTCCTCCGCACC ATACCCAATGATGAA CACCAGGCCACGGCC ATGGCTGACATCATC
1140 GAGTACTTCCGCTGG AACTGGGTGGGCACA ATTGCAGCTGACGAT GACTATGGCCGGCCA
1200 GGGATCGAGAAGTTT CGAGAGGAAGCTGAG GAGAGGGACATCTGC ATCGACTTCAGCGAG
1260 CTCATCTCCCAATAC TCTGATGAGGAAAAG ATCCAGCAGGTGGTG GAGGTGATCCAGAAT
1320 TCCACCGCCAAAGTC ATTGTCCGTCTTCTC CAGCGGCCCAGACCT GGAACCCCTCATCAA
1380 AGAGATCGTCCGGCG CAATATCACAGGCAG GATCTGGCTGGCCAG CGAGSCTGGSCAGCT
1440 CTTCCCTGATTGCTA TGCCCGAGTATTCC ATGTGGTCGGAGGCA CCATTGGGTTTGGTT
1500 TGAAAGCTGGGCAGA TCCCAGGCTTCCGGG AATTCTGCAGAAAG TCCACCCCAGGAAGT
1560 CTGTCCACAATGGTT TTGCCAAGGAGTTTT GGGAAGAAACATTTA ACTGCCACCTGCAAG
1620 AGGGTGCTAAAGGCC CATTACCGGTGGACA CCTTCCTGAGAGGTC ACGAAGAAGGAGGTG
1680 CCAGGTTAAGCAACA GTCCCACTGCCTTCC GACCTCTGTGCACTG GGGAGGAGAACATCA
1740 GCAGTGTCGAGACTC CTTACATGGATTATA CACATTTACGGATAT CCTACAACGTCTACT
1800 TAGCCGTCTACTCCA TTGCTCATGCCCTAC AAGATATATACACCT GCATACCTGGGAGAG
1860 GGCTCTTCACCAACG GTTCCTGCGCAGATA TCAAGAAGGTTGAAG CTTGGCAGGTCCTGA
1920 AACACCTGCGGCACC TAAATTTTACCAGCA ATATGGGGGAGCAAG TAACTTTCGATGAAT
1980 GTGGAGACCTGGCAG GGAACTATTCCATCA TCAACTGGCACCTCT CCCCAGAGGACGGCT
2040 CCATAGTGTTTAAGG AAGTTGGATATTACA ATGTCTATGCCAAGA AAGGAGAGAGACTCT
2100 TCATCAATGATGAAA AAATTCTGTGGAGTG GATTCTCAAGGGAGG TGC 2148
```

FIG. 47

ERG/lfr
3/9/93
8985V/*

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Brigham and Women's Hospital
                   75 Francis Street
                   Boston, Massachusetts USA 02115

                   NPS Pharmaceuticals, Inc.
                   420 Chipeta Way
                   Salt Lake City, Utah USA 84108

Inventors: NEMETH, Edward F. et al.

International
Serial No: PCT/US93/01642
Filing Date: 23 February 1993
For: Calcium Receptor-Active Molecules

BOX PCT
Hon. Commissioner of Patents and Trademarks
Washington, D. C.   20231

Sir:

REMARKS

Enclosed are substitute pages 9, 41, and 166 in connection with the above-identified patent application. Also enclosed is the American Type Culture Collection (ATCC), Rockville, Maryland receipt indicating that the deposit made in connection with the instant application was received on 19 February 1993 and was assigned ATCC number 75416.

The undersigned attorney had not yet been informed by the ATCC as to the particular deposit number at the time of filing the application. Accordingly, substitute pages including the correct number are enclosed.

Respectfully submitted,

Edward R. Gates, Reg. No. 31,616
Wolf, Greenfield & Sacks, P.C.
600 Atlantic Avenue
Boston, MA 02210
Tel. (617)720-3500
Attorney for Applicant

A. CLASSIFICATION OF SUBJECT MATTER

IPC 5	A61K31/00	A61K31/13	A61K31/135	A61K31/405	C07K15/00
	C12N15/12	C12N15/11	C12N5/10	C07K13/00	C07K15/28
	A01K67/027				

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5   A61K   C07K   C12N   A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E	WO,A,93 04373 (NPS PHARMACEUTICALS INC.) 4 March 1993 see the whole document ---	1-82,89, 98-103
X	EP,A,0 253 327 (CHINOIN) 20 January 1988 see the whole document ---	1-23, 25-27
X	WO,A,91 00853 (NEW YORK UNIVERSITY) 24 January 1991 see claims ---	1-23
X	WO,A,92 14709 (CAMBRIDGE NEUROSCIENCE INC.) 3 September 1992 see claims --- -/--	1-23

[X] Further documents are listed in the continuation of box C.	[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 November 1993	23. 12. 93

Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Authorized officer KLAVER, T

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 395 357 (PFIZER INC.) 31 October 1990 see claims ---	1-23,36, 38,39
X	GB,A,2 113 089 (UNIVERSITY OF NEW YORK) 3 August 1983 * whole document, esp. page 2 * ---	1-24
A	BIOSCIENCE REP. vol. 10, no. 6 , 1990 pages 493 - 507 M. ZAIDI '"Calcium receptors" on eukaryotic cells with special reference to the osteoclast.' cited in the application ---	
A	CELL CALCIUM vol. 11, no. 5 , 1990 pages 319 - 321 E.F. NEMETH ET AL. 'The role of extracellular calcium in the regulation of intracellular calcium and cell function.' cited in the application ---	
A	CELL CALCIUM vol. 11, no. 5 , 1990 pages 323 - 327 E.F. NEMETH 'Regulation of cytosolic calcium by extracellular divalent cations in C-cells and parathyroid cells.' cited in the application ---	
A	NORMAN, VANAMAN, MEANS , EDS. 'calcium-binding proteins in health and disease' 1987 , ACADEMIC PRESS , SAN DIEGO Evidence for the presence of a novel calcium-binding protein calcium receptor on the surface of parathyroid cells. Auth. E.F. Nemeth see page 36 - page 38 -----	

2

Box I   Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
    because they relate to subject matter not required to be searched by this Authority, namely:
    REMARK: Although claims 36-89 are directed to a method of treatment of or a

    diagnostic method practised on the human/animal body the search has been ca
    rried out based on the alleged effects of the compound/composition.

2. [X] Claims Nos.:
    because they relate to parts of the international application that do not comply with the prescribed requirements to such
    an extent that no meaningful international search can be carried out, specifically:
    Claims searched incompletely: 1-22,25,27,36-60,63,65,83.
    For further information see annex.

3. [ ] Claims Nos.:
    because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all
    searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment
    of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report
    covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is
    restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   [ ] The additional search fees were accompanied by the applicant's protest.

                               [ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM   PCT/ISA/206

a. A compound cannot be sufficiently described by its psycho-chemical or pharmachological characteristics. The search has therefor been restricted to compounds mentioned in the claims and the description (cl.1-22,36-60).

b. In view of the large number of compounds defined by the general formula of claims 25,27,63 and 65 the search has been limited to the inventive part of the molecule and/or the compounds mentioned in the description and/or in the claims (PCT Art. 6).

c. The subject matter of claim 83 is not clear, because the actual matter is not revealed.